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**PATENT**

Attorney Docket No. AMBER-06797

**THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: Rothschild *et al.*

Serial No.: 10/049,332

Group No.:

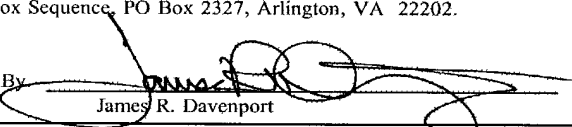
Filed: 02/11/02

Examiner:

Entitled: **Methods For The Detection, Analysis And  
Isolation Of Nascent Proteins**

**PRELIMINARY AMENDMENT AND RESPONSE  
TO NOTIFICATION OF MISSING REQUIREMENTS  
UNDER 35 USC 371 IN THE UNITED STATES  
DESIGNATED/ELECTED OFFICE  
MAILED APRIL 16, 2002**

US Patent and Trademark Office  
Box Sequence, PO Box 2327  
Arlington, VA 22202

<b>CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)</b>	
I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: US Patent and Trademark Office, Box Sequence, PO Box 2327, Arlington, VA 22202.	
Dated. <u>June 17, 2002</u>	By  James R. Davenport

Sir:

This is responsive to the Notification of Missing Requirements Under 35 USC 371 in the United States Designated/Elected Office mailed on April 16, 2002. Prior to examination of the instant application, please amend the application as follows.

Instructions to amend the specification, and replacement or added paragraphs in clean form pursuant to 37 C.F.R. §1.121(b) is included beginning on page 2 of this communication. A marked-up version of the specification's replacement paragraphs pursuant to 37 C.F.R. §1.121(b) is attached as Appendix I.

**PATENT**

Attorney Docket No. AMBER-06797

**CLEAN VERSION OF REPLACEMENT PARAGRAPHS  
IN THE SPECIFICATION PURSUANT TO 37 C.F.R. §1.121(b)**

**IN THE SPECIFICATION**

On page 19, please delete the paragraphs beginning on line 21 and ending on line 23, and replace with the following paragraphs:

Figure 1 shows the structure of (A) an amino acid and (B) a peptide (SEQ ID NO:37).

Figure 2 provides a description of the molecular steps that occur during protein synthesis in a cellular or cell-free system (SEQ ID NOS:38-40).

Please insert the attached Sequence Listing as new pages --140-151--.

**IN THE FIGURES**

Please replace Table 4 with the attached Table 4 Substitute Sheet.

**IN THE CLAIMS**

Please renumber the Claims pages from pages "140-149" to --152-161--.

**REMARKS**

The specification and figure have been amended to provide sequence identifiers. Applicants' amendments do not introduce new matter.

The Examiner has requested that a Sequence Listing be provided. Applicants submit this Amendment and Response to provide as a separate part of the disclosure, a "Sequence Listing" pursuant to 37 C.F.R. §§ 1.821-1.825. Applicants submit herewith in paper copy and on floppy disk the Sequence Listing in computer readable form. The contents of the paper and computer readable copies are the same and include no new matter.

Applicants respectfully request entry of this Preliminary Amendment prior to examination of the present application.

WO 01/14578

PCT/US00/23233

### Various Epitopes and Their Sequences

Name	Amino acid sequence	Nucleotide sequence (5'→ 3')
His-6	HHHHHH (SEQ ID NO:5)	CATCACCATCACCATCAC (SEQ ID NO:27)
FLAG	DYKDDDDK (SEQ ID NO:7)	GACTACAAGGACGACGACAAG (SEQ ID NO:28)
c-Myc	EQKLISEEDL (SEQ ID NO:6)	GAGCAGAAGCTGATCAGCGAGGAGGACCTG (SEQ ID NO:29)
Strep-Tag	WSHPQFEK (SEQ ID NO:8)	TGGAGCCACCCCCAGTTCGAGAAG (SEQ ID NO:30)
Amber-16	CSPFEVQVSPEA GAQK (SEQ ID NO:22)	TGCAGCCCCCTTCGAGGTGCAGGTGAGCCCCGAGCCGGCGCC CAGAAG (SEQ ID NO:31)
T7-Tag	MASMTGGQQMG (SEQ ID NO:23)	ATGGCCAGCATGACCGGCGGCCAGCAGATGGGC (SEQ ID NO:32)
VSV-G <sup>#</sup> -Tag	YTDIEMNRLGK (SEQ ID NO:24)	TACACCGACATCGAGATGAACCGCCTGGGCAAG (SEQ ID NO:33)
HA <sup>*</sup> -Tag	YPYDVPDYA (SEQ ID NO:9)	TACCCCTACGACGTGCCCGACTACGCC (SEQ ID NO:34)
Protein-C Tag	EDQVDPRLIDGK (SEQ ID NO:25)	GAGGACCAGGTGGACCCCCGCCTGATCGACGGCAAG (SEQ ID NO:35)
HSV <sup>\$</sup> -Tag	QPELAPEDPED (SEQ ID NO:26)	CAGCCCGAGCTGGCCCCCGAGGACCCCGAGGAC (SEQ ID NO:36)

# - Vesicular Stomatitis Virus Glycoprotein

\* - HemAgglutinin

\$ - Herpes Simplex Virus glycoprotein

## TABLE 4

1004-9332-062102

**PATENT**

Attorney Docket No. AMBER-06797

Dated: June 17, 2002

Christine A. Lekutis/

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**APPENDIX I**

**MARKED-UP VERSION OF SPECIFICATION'S REPLACEMENT PARAGRAPHS  
AND REWRITTEN, ADDED, AND/OR CANCELLED CLAIMS**

The following is a marked-up version of the specification's replacement paragraphs pursuant to 37 C.F.R. §1.121(b) with markings showing changes made herein to the previous version of record of the specification.

**IN THE SPECIFICATION**

On page 19, please delete the paragraphs beginning on line 21 and ending on line 23, and replace with the following paragraphs:

Figure 1 shows the structure of (A) an amino acid and (B) a peptide (SEQ ID NO:37).

Figure 2 provides a description of the molecular steps that occur during protein synthesis in a cellular or cell-free system (SEQ ID NOS:38-40).

WO 01/14578

PCT/US00/23233

# Various Epitopes and Their Sequences

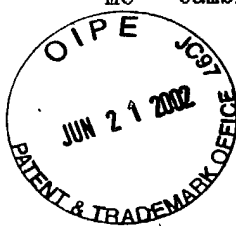
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# - Vesicular Stomatitis Virus Glycoprotein

\* - HemAgglutinin

\$ - Herpes Simplex Virus glycoprotein

## TABLE 4

**PATENT**

Attorney Docket No. AMBER-06797

Applicant / Patentee: Kenneth J. Rothschild *et al.*  
For: Methods For The Detection, Analysis And Isolation Of Nascent Proteins

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**(37 CFR § 1.9(c) - SMALL BUSINESS CONCERN)**

I hereby declare that I am an official of the small business concern empowered to act on behalf of the concern identified below:

AMBERGEN, INC.  
1106 Commonwealth Avenue, Boston, MA 02215

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR §§ 121.3-18, and reproduced in 37 CFR § 1.9(d), for purposes of paying reduced fees under §§ 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **Methods For The Detection, Analysis And Isolation Of Nascent Proteins** by inventors named **Kenneth J. Rothschild *et al.***, described in the specification filed herewith.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date:

5-23-02

By:

Name:

Kenneth Rothschild

Title:

Ambergen President

**METHODS FOR THE DETECTION, ANALYSIS AND**  
**ISOLATION OF NASCENT PROTEINS**

**Field of the Invention**

This invention relates to non-radioactive markers that facilitate the detection and analysis of nascent proteins translated within cellular or cell-free translation systems. Nascent proteins containing these markers can be rapidly and efficiently detected, isolated and analyzed without the handling and disposal problems associated with radioactive reagents.

**Background of the Invention**

Cells contain organelles, macromolecules and a wide variety of small molecules. Except for water, the vast majority of the molecules and macromolecules can be classified as lipids, carbohydrates, proteins or nucleic acids. Proteins are the most abundant cellular components and facilitate many of the key cellular processes. They include enzymes, antibodies, hormones, transport molecules and components for the cytoskeleton of the cell.

Proteins are composed of amino acids arranged into linear polymers or polypeptides. In living systems, proteins comprise over twenty common amino acids. These twenty or so amino acids are generally termed the native amino acids. At the center of every amino acid is the alpha carbon atom ( $C\alpha$ ) which forms four bonds or attachments with other molecules (Figure 1). One bond is a covalent linkage to an amino group ( $NH_2$ ) and another to a carboxyl group ( $COOH$ ) which both participate in polypeptide formation. A third bond is nearly always linked to a hydrogen atom and the fourth to a side chain which imparts variability to the amino acid structure. For example, alanine is formed when the side chain is a methyl group ( $-CH_3$ ) and a valine is formed when the side chain is an isopropyl group ( $-CH(CH_3)_2$ ). It is also possible to chemically synthesize amino acids containing different side-chains, however, the cellular protein synthesis system, with rare exceptions, utilizes native amino acids. Other amino acids and structurally similar chemical compounds are termed non-native

and are generally not found in most organisms.

A central feature of all living systems is the ability to produce proteins from amino acids. Basically, protein is formed by the linkage of multiple amino acids via peptide bonds such as the pentapeptide depicted in Figure 1B. Key molecules  
5 involved in this process are messenger RNA (mRNA) molecules, transfer RNA (tRNA) molecules and ribosomes (rRNA-protein complexes). Protein translation normally occurs in living cells and in some cases can also be performed outside the cell in systems referred to as cell-free translation systems. In either system, the basic process of protein synthesis is identical. The extra-cellular or cell-free translation system  
10 comprises an extract prepared from the intracellular contents of cells. These preparations contain those molecules which support protein translation and depending on the method of preparation, post-translational events such as glycosylation and cleavages as well. Typical cells from which cell-free extracts or *in vitro* extracts are made are *Escherichia coli* cells, wheat germ cells, rabbit reticulocytes, insect cells and  
15 frog oocytes.

Both *in vivo* and *in vitro* syntheses involve the reading of a sequence of bases on a mRNA molecule. The mRNA contains instructions for translation in the form of triplet codons. The genetic code specifies which amino acid is encoded by each triplet codon. For each codon which specifies an amino acid, there normally exists a cognate  
20 tRNA molecule which functions to transfer the correct amino acid onto the nascent polypeptide chain. The amino acid tyrosine (Tyr) is coded by the sequence of bases UAU and UAC, while cysteine (Cys) is coded by UGU and UGC. Variability associated with the third base of the codon is common and is called wobble.

Translation begins with the binding of the ribosome to mRNA (Figure 2). A  
25 number of protein factors associate with the ribosome during different phases of translation including initiation factors, elongation factors and termination factors. Formation of the initiation complex is the first step of translation. Initiation factors contribute to the initiation complex along with the mRNA and initiator tRNA (fmet and met) which recognizes the base sequence UAG. Elongation proceeds with charged  
30 tRNAs binding to ribosomes, translocation and release of the amino acid cargo into the

peptide chain. Elongation factors assist with the binding of tRNAs and in elongation of the polypeptide chain with the help of enzymes like peptidyl transferase. Termination factors recognize a stop signal, such as the base sequence UGA, in the message terminating polypeptide synthesis and releasing the polypeptide chain and the mRNA from the ribosome.

The structure of tRNA is often shown as a cloverleaf representation (Figure 3A). Structural elements of a typical tRNA include an acceptor stem, a D-loop, an anticodon loop, a variable loop and a TΨC loop. Aminoacylation or charging of tRNA results in linking the carboxyl terminal of an amino acid to the 2'-(or 3'-) hydroxyl group of a terminal adenosine base via an ester linkage. This process can be accomplished either using enzymatic or chemical methods. Normally a particular tRNA is charged by only one specific native amino acid. This selective charging, termed here enzymatic aminoacylation, is accomplished by aminoacyl tRNA synthetases. A tRNA which selectively incorporates a tyrosine residue into the nascent polypeptide chain by recognizing the tyrosine UAC codon will be charged by tyrosine with a tyrosine-aminoacyl tRNA synthetase, while a tRNA designed to read the UGU codon will be charged by a cysteine-aminoacyl tRNA synthetase. These synthetases have evolved to be extremely accurate in charging a tRNA with the correct amino acid to maintain the fidelity of the translation process. Except in special cases where the non-native amino acid is very similar structurally to the native amino acid, it is necessary to use means other than enzymatic aminoacylation to charge a tRNA.

Molecular biologists routinely study the expression of proteins that are coded for by genes. A key step in research is to express the products of these genes either in intact cells or in cell-free extracts. Conventionally, molecular biologists use radioactively labeled amino acid residues such as <sup>35</sup>S-methionine as a means of detecting newly synthesized proteins or so-called nascent proteins. These nascent proteins can normally be distinguished from the many other proteins present in a cell or a cell-free extract by first separating the proteins by the standard technique of gel electrophoresis and determining if the proteins contained in the gel possess the specific radioactively labeled amino acids. This method is simple and relies on gel

electrophoresis, a widely available and practiced method. It does not require prior knowledge of the expressed protein and in general does not require the protein to have any special properties. In addition, the protein can exist in a denatured or unfolded form for detection by gel electrophoresis. Furthermore, more specialized techniques such as blotting to membranes and coupled enzymatic assays are not needed.

Radioactive assays also have the advantage that the structure of the nascent protein is not altered or can be restored, and thus, proteins can be isolated in a functional form for subsequent biochemical and biophysical studies.

Radioactive methods suffer from many drawbacks related to the utilization of radioactively labeled amino acids. Handling radioactive compounds in the laboratory always involves a health risk and requires special laboratory safety procedures, facilities and detailed record keeping as well as special training of laboratory personnel. Disposal of radioactive waste is also of increasing concern both because of the potential risk to the public and the lack of radioactive waste disposal sites. In addition, the use of radioactive labeling is time consuming, in some cases requiring as much as several days for detection of the radioactive label. The long time needed for such experiments is a key consideration and can seriously impede research productivity. While faster methods of radioactive detection are available, they are expensive and often require complex image enhancement devices.

The use of radioactive labeled amino acids also does not allow for a simple and rapid means to monitor the production of nascent proteins inside a cell-free extract without prior separation of nascent from preexisting proteins. However, a separation step does not allow for the optimization of cell-free activity. Variables including the concentration of ions and metabolites and the temperature and the time of protein synthesis cannot be adjusted.

Radioactive labeling methods also do not provide a means of isolating nascent proteins in a form which can be further utilized. The presence of radioactivity compromises this utility for further biochemical or biophysical procedures in the laboratory and in animals. This is clear in the case of *in vitro* expression when proteins cannot be readily produced *in vivo* because the protein has properties which

are toxic to the cell. A simple and convenient method for the detection and isolation of nascent proteins in a functional form could be important in the biomedical field if such proteins possessed diagnostic or therapeutic properties. Recent research has met with some success, but these methods have had numerous drawbacks.

5           Radioactive labeling methods also do not provide a simple and rapid means of detecting changes in the sequence of a nascent protein which can indicate the presence of potential disease causing mutations in the DNA which code for these proteins or fragments of these proteins. Current methods of analysis at the protein level rely on the use of gel electrophoresis and radioactive detection which are slow and not  
10           amenable to high throughput analysis and automation. Such mutations can also be detected by performing DNA sequence analysis on the gene coding for a particular protein or protein fragment. However, this requires large regions of DNA to be sequenced, which is time-consuming and expensive. The development of a general method which allows mutations to be detected at the nascent protein level is  
15           potentially very important for the biomedical field.

          Radioactive labeling methods also do not provide a simple and rapid means of studying the interaction of nascent proteins with other molecules including compounds which might be have importance as potential drugs. If such an approach were available, it could be extremely useful for screening large numbers of compounds  
20           against the nascent proteins coded for by specific genes, even in cases where the genes or protein has not yet been characterized. In current technology, which is based on affinity electrophoresis for screening of potential drug candidates, both in natural samples and synthetic libraries, proteins must first be labeled uniformly with a specific marker which often requires specialized techniques including isolation of the protein  
25           and the design of special ligand markers or protein engineering.

          Special tRNAs, such as tRNAs which have suppressor properties, suppressor tRNAs, have been used in the process of site-directed non-native amino acid replacement (SNAAR) (C. Noren et al., Science 244:182-188, 1989). In SNAAR, a unique codon is required on the mRNA and the suppressor tRNA, acting to target a  
30           non-native amino acid to a unique site during the protein synthesis (PCT



WO90/05785). However, the suppressor tRNA must not be recognizable by the aminoacyl tRNA synthetases present in the protein translation system (Bain et al., Biochemistry 30:5411-21, 1991). Furthermore, site-specific incorporation of non-native amino acids is not suitable in general for detection of nascent proteins in a cellular or cell-free protein synthesis system due to the necessity of incorporating non-sense codons into the coding regions of the template DNA or the mRNA.

Products of protein synthesis may also be detected by using antibody based assays. This method is of limited use because it requires that the protein be folded into a native form and also for antibodies to have been previously produced against the nascent protein or a known protein which is fused to the unknown nascent protein. Such procedures are time consuming and again require identification and characterization of the protein. In addition, the production of antibodies and amino acid sequencing both require a high level of protein purity.

In certain cases, a non-native amino acid can be formed after the tRNA molecule is aminoacylated using chemical reactions which specifically modify the native amino acid and do not significantly alter the functional activity of the aminoacylated tRNA (Promega Technical Bulletin No. 182; tRNA<sup>nscond</sup>™: Non-radioactive Translation Detection System, Sept. 1993). These reactions are referred to as post-aminoacylation modifications. For example, the  $\epsilon$ -amino group of the lysine linked to its cognate tRNA (tRNA<sup>LYS</sup>), could be modified with an amine specific photoaffinity label (U.C. Krieg et al., Proc. Natl. Acad. Sci. USA 83:8604-08, 1986). These types of post-aminoacylation modifications, although useful, do not provide a general means of incorporating non-native amino acids into the nascent proteins. The disadvantage is that only those non-native amino acids that are derivatives of normal amino acids can be incorporated and only a few amino acid residues have side chains amenable to chemical modification. More often, post-aminoacylation modifications can result in the tRNA being altered and produce a non-specific modification of the  $\alpha$ -amino group of the amino acid (e.g. in addition to the  $\epsilon$ -amino group) linked to the tRNA. This factor can lower the efficiency of incorporation of the non-native amino acid linked to the tRNA. Non-specific, post-aminoacylation modifications of tRNA

structure could also compromise its participation in protein synthesis. Incomplete chain formation could also occur when the  $\alpha$ -amino group of the amino acid is modified.

In certain other cases, a nascent protein can be detected because of its special and unique properties such as specific enzymatic activity, absorption or fluorescence. This approach is of limited use since most proteins do not have special properties with which they can be easily detected. In many cases, however, the expressed protein may not have been previously characterized or even identified, and thus, its characteristic properties are unknown.

## Summary of the Invention

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides methods for the labeling, detection, quantitation, analysis and isolation of nascent proteins produced in a cell-free or cellular translation system without the use of radioactive amino acids or other radioactive labels. One embodiment of the invention is directed to methods for detecting nascent proteins translated in a translation system. A tRNA molecule is aminoacylated with a fluorescent marker to create a misaminoacylated tRNA. The misaminoacylated, or charged, tRNA can be formed by chemical, enzymatic or partly chemical and partly enzymatic techniques which place a fluorescent marker into a position on the tRNA molecule from which it can be transferred into a growing peptide chain. Markers may comprise native or non-native amino acids with fluorescent moieties, amino acid analogs or derivatives with fluorescent moieties, detectable labels, coupling agents or combinations of these components with fluorescent moieties. The misaminoacylated tRNA is introduced to the translation system such as a cell-free extract, the system is incubated and the fluorescent marker incorporated into nascent proteins.

It is not intended that the present invention be limited to the nature of the particular fluorescent moiety. A variety of fluorescent compounds are contemplated, including fluorescent compounds that have been derivatized (e.g. with NHS) to be



The present invention contemplates aminoacylation of the tRNA molecule by chemical or enzymatic misaminoacylation. The present invention also contemplates embodiments wherein two or more different misaminoacylated tRNAs are introduced into the translation system. In a preferred embodiment, the nascent protein detected (by virtue of the incorporated marker) is functionally active.

It is not intended that the present invention be limited by the particular nature of the nascent protein. In one embodiment, the present invention contemplates a method for detecting nascent proteins which are conjugated to the mRNA message which codes for all or part of the nascent protein. In general, a variety of modifications of the nascent protein are envisioned including post-translational modifications, proteolysis, attachment of an oligonucleotide through a puromycin linker to the C-terminus of the protein, and interaction of the nascent protein with other components of the translation system including those which are added exogenously.

It is not intended that the present invention be limited by the particular nature of the tRNA molecule. In one embodiment, the tRNA molecule is an initiator tRNA molecule. In another embodiment, the tRNA molecule is a suppressor tRNA molecule.

The present invention also contemplates kits. In one embodiment, the kit comprises a) a first containing means (e.g. tubes, vials, etc) containing at least one component of a protein synthesis system; and b) a second containing means containing a misaminoacylated tRNA, wherein said tRNA is misaminoacylated with a BODIPY marker. Such kits may include initiator tRNA and/or suppressor tRNA. Importantly, the kit is not limited to the particular components of said protein synthesis system; a variety of components are contemplated (e.g. ribosomes).

Another embodiment of the invention is directed to methods for detecting nascent proteins translated in a translation system. A tRNA molecule is aminoacylated with one component of a binary marker system. The misaminoacylated, or charged, tRNA can be formed by chemical, enzymatic or partly chemical and partly enzymatic techniques which place a component of a binary marker system into a position on the tRNA molecule from which it can be transferred into a growing peptide chain. The

component of the binary marker system may comprise native or non-native amino acids, amino acid analogs or derivatives, detectable labels, coupling agents or combinations of these components. The misaminoacylated tRNA is introduced to the translation system such as a cell-free extract, the system is incubated and the marker  
5 incorporated into nascent proteins. The second component of the binary marker system is then introduced making the first component incorporated into the nascent protein specifically detectable. These methods may be used to detect, isolate and quantitate such nascent proteins as recombinant gene products, gene fusion products, enzymes, cytokines, hormones, immunogenic proteins, human proteins, carbohydrate and lipid  
10 binding proteins, nucleic acid binding proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.

It is not intended that the present invention be limited to a particular translation system. In one embodiment, a cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit  
15 reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof. It is also not intended that the present invention be limited to the particular reaction conditions employed. However, typically the cell-free translation system is incubated at a temperature of between about 25°C to about 45°C. The present invention  
20 contemplates both continuous flow systems or dialysis systems.

Another embodiment of the invention is directed to methods for the detection of nascent proteins translated in a cellular or cell-free translation system using non-radioactive markers which have detectable electromagnetic spectral properties. As  
25 before, a non-radioactive marker is misaminoacylated to a tRNA molecule and the misaminoacylated tRNA is added to the translation system. The system is incubated to incorporate marker into the nascent proteins. Nascent proteins containing marker can be detected from the specific electromagnetic spectral property of the marker. Nascent proteins can also be isolated by taking advantage of unique properties of these markers or by conventional means such as electrophoresis, gel filtration, high-pressure or fast-  
30 pressure liquid chromatography, affinity chromatography, ion exchange

chromatography, chemical extraction, magnetic bead separation, precipitation or combinations of these techniques.

Another embodiment of the invention is directed to the synthesis of nascent proteins containing markers which have reporter properties when the reporter is brought into contact with a second agent. Reporter markers are chemical moieties which have detectable electromagnetic spectral properties when incorporated into peptides and whose spectral properties can be distinguished from unincorporated markers and markers attached to tRNA molecules. As before, tRNA molecules are misaminoacylated, this time using reported markers. The misaminoacylated tRNAs are added to a translation system and incubated to incorporate marker into the peptide. Reporter markers can be used to follow the process of protein translation and to detect and quantitate nascent proteins without prior isolation from other components of the protein synthesizing system.

Another embodiment of the invention is directed to compositions comprised of nascent proteins translated in the presence of markers, isolated and, if necessary, purified in a cellular or cell-free translation system. Compositions may further comprise a pharmaceutically acceptable carrier and be utilized as an immunologically active composition such as a vaccine, or as a pharmaceutically active composition such as a drug, for use in humans and other mammals.

Another embodiment of the invention is directed to methods for detecting nascent proteins translated in a translation system by using mass spectrometry. A non-radioactive marker of known mass is misaminoacylated to a tRNA molecule and the misaminoacylated tRNA is added to the translation system. The system is incubated to incorporate the mass marker into the nascent proteins. The mass spectrum of the translation system is then measured. The presence of the nascent protein can be directly detected by identifying peaks in the mass spectrum of the protein synthesis system which correspond to the mass of the unmodified protein and a second band at a higher mass which corresponds to the mass of the nascent protein plus the modified amino acid containing the mass of the marker. When the mass marker is photocleavable, the assignment of the second band to a nascent protein containing the

mass marker can be verified by removing the marker with light.

Another embodiment of the invention is directed to methods for detecting nascent proteins with mutations which are translated in a translation system. RNA or DNA coding for the protein which may contain a possible mutation is added to the translation system. The system is incubated to synthesize the nascent proteins. The nascent protein is then separated from the translation system using an affinity marker located at or close to the N-terminal end of the protein. The protein is then analyzed for the presence of a detectable marker located at or close to the N-terminal of the protein (N-terminal marker). A separate measurement is then made on a sequence dependent detectable marker located at or close to the C-terminal end of the protein (C-terminal marker). A comparison is then made of the level of incorporation of the N-terminal and C-terminal markers in the nascent protein. It is not intended that the present invention be limited by the nature of the N- and C-terminal markers, or the type of affinity marker utilized. A variety of markers are contemplated. In one embodiment, the affinity marker comprises an epitope recognized by an antibody or other binding molecule. In one embodiment, the N-terminal marker comprises a fluorescent marker (e.g. a BODIPY marker), while the C-terminal marker comprises a metal binding region (e.g. His tag).

The present invention contemplates a variety of methods wherein the three markers (e.g. the N- and C-terminal markers and the affinity markers) are introduced into a nascent protein. In one embodiment, the method comprises: a) providing i) a misaminoacylated initiator tRNA molecule which only recognizes the first AUG codon that serves to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising a C-terminal marker and (in some embodiments) an affinity marker; b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker, said C-terminal marker and (in some embodiments) said affinity marker. In one embodiment, the method further comprises, after step b), isolating said nascent protein.

In another embodiment, the method comprises: a) providing i) a misaminoacylated initiator tRNA molecule which only recognizes the first AUG codon that serves to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising a C-terminal marker and (in some embodiments) an affinity marker; b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker at the N-terminus of said protein, a C-terminal marker, and (in some embodiments) said affinity marker adjacent to said first marker.

10 In one embodiment, the method further comprises, after step b), isolating said nascent protein.

In yet another embodiment, the method comprises: a) providing i) a misaminoacylated tRNA molecule which only recognizes the first codon designed to serve to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising a C-terminal marker and (in some embodiments) an affinity marker; b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker, said C-terminal marker and (in some

15 20 embodiments) said affinity marker. In one embodiment, the method further comprises, after step b), isolating said nascent protein.

In still another embodiment, the method comprises: a) providing i) a misaminoacylated tRNA molecule which only recognizes the first codon designed to serve to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising a C-terminal marker and (in some embodiments) an affinity marker; b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker at the N-terminus of said protein, a C-terminal marker, and (in some embodiments) said affinity marker adjacent to said first marker.

25 30



In one embodiment, the method further comprises, after step b), isolating said nascent protein.

The present invention also contemplates embodiments where only two markers are employed (e.g. a marker at the N-terminus and a marker at the C-terminus). In one embodiment, the nascent protein is non-specifically bound to a solid support (e.g. beads, microwells, strips, etc.), rather than by the specific interaction of an affinity marker. In this context, "non-specific" binding is meant to indicate that binding is not driven by the uniqueness of the sequence of the nascent protein. Instead, binding can be by charge interactions. In one embodiment, the present invention contemplates that the solid support is modified (e.g. functionalized to change the charge of the surface) in order to capture the nascent protein on the surface of the solid support. In one embodiment, the solid support is poly-L-lysine coated. In yet another embodiment, the solid support is nitrocellulose (e.g. strips, microcellulose containing microwells, etc.). Regardless of the particular nature of the solid support, the present invention contemplates that the nascent protein containing the two markers is captured under conditions that permit the ready detection of the markers.

In both the two marker and three marker embodiments described above, the present invention contemplates that one or more of the markers will be introduced into the nucleic acid template by primer extension or PCR. In one embodiment, the present invention contemplates a primer comprising (on or near the 5'-end) a promoter, a ribosome binding site ("RBS"), and a start codon (e.g. ATG), along with a region of complementarity to the template. In another embodiment, the present invention contemplates a primer comprising (on or near the 5'-end) a promoter, a ribosome binding site ("RBS"), a start codon (e.g. ATG), a region encoding an affinity marker, and a region of complementarity to the template. It is not intended that the present invention be limited by the length of the region of complementarity; preferably, the region is greater than 8 bases in length, more preferably greater than 15 bases in length, and still more preferably greater than 20 bases in length.

It is also not intended that the present invention be limited by the ribosome binding site. In one embodiment, the present invention contemplates primers

comprising the *Kozak sequence*, a string of non-random nucleotides (consensus sequence 5'-GCCA/GCCATGG-3') (SEQ ID NO:1) which are present before the translation initiating *first* ATG in majority of the mRNAs which are transcribed and translated in an eukaryotic cells. See M. Kozak, *Cell* 44:283-292 (1986). In another embodiment, the present invention contemplates a primer comprising the the  
5 prokaryotic mRNA ribosome binding site, which usually contains part or all of a polypurine domain UAAGGAGGU (SEQ ID NO:2) known as the Shine-Dalgarno (SD) sequence found just 5' to the translation initiation codon: mRNA 5'-UAAGGAGGU - N<sub>5-10</sub> - AUG. (SEQ ID NO:3)

10 For PCR, two primers are used. In one embodiment, the present invention contemplates as the forward primer a primer comprising (on or near the 5'-end) a promoter, a ribosome binding site ("RBS"), and a start codon (e.g. ATG), along with a region of complementarity to the template. In another embodiment, the present invention contemplates as the forward primer a primer comprising (on or near the 5'-  
15 end) a promoter, a ribosome binding site ("RBS"), a start codon (e.g. ATG), a region encoding an affinity marker, and a region of complementarity to the template. The present invention contemplates that the reverse primer, in one embodiment, comprises (at or near the 5'-end) one or more stop condons and a region encoding a C-terminus marker (such as a HIS-tag).

20 Another embodiment of the invention is directed to methods for detecting by electrophoresis (*e.g.* capillary electrophoresis) the interaction of molecules with nascent proteins which are translated in a translation system. A tRNA misaminoacylated with a detectable marker is added to the protein synthesis system. The system is incubated to incorporate the detectable marker into the nascent proteins. One or more specific  
25 molecules are then combined with the nascent proteins (either before or after isolation) to form a mixture containing nascent proteins/molecule conjugates. Aliquots of the mixture are then subjected to capillary electrophoresis. Nascent proteins/molecule conjugates are identified by detecting changes in the electrophoretic mobility of nascent proteins with incorporated markers.

30 The present invention also contemplates using the above described methods and

compositions in a method for drug/proteome screening. For example, the present invention contemplates a method of identifying a protein or portion of a protein which interacts with a ligand (such as a drug), which method comprises the steps of preparing a cDNA library from a cell that expresses said desired protein; inserting said cDNA library into an expression vector, thereby forming an expression cDNA library; transforming said expression library into bacterial cells and culturing said bacterial cells to produce individual bacterial colonies, wherein each colony contains a member of said expression library; collecting pools of a predetermined number of said individual bacterial colonies; isolating cDNA from said pools of bacterial colonies; expressing proteins (or portions of proteins) encoded by said cDNAs; and identifying a desired protein (or portion thereof) and the cDNA encoding said desired protein (or portion); wherein pools of approximately 50 individual bacterial colonies are collected (more preferably less than 50, still more preferably less than 40, still more preferably less than 30, and most preferably 25 or less, but more than 10), wherein each colony contains a member of an expression cDNA library, thereby forming cDNA pools; and wherein proteins (or portions thereof) encoded by the cDNA in said cDNA pools are expressed in an *in vitro* transcription/translation system in a common reaction mixture, and wherein a desired protein (or portion thereof) is identified from said reaction mixture.

Other embodiments and advantages of the invention are set forth, in part, in the description which follows and, in part, will be obvious from this description, or may be learned from the practice of the invention.

### Definitions

To facilitate understanding of the invention, a number of terms are defined below.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may have 5' and 3' ends.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

A primer is selected to have on its 3' end a region that is "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to

occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

As used herein, the terms "hybridize" and "hybridization" refers to the annealing of a complementary sequence to the target nucleic acid. The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. Marmur and Lane, Proc. Natl. Acad. Sci. USA 46:453 (1960) and Doty *et al.*, Proc. Natl. Acad. Sci. USA 46:461 (1960). The terms "annealed" and "hybridized" are used interchangeably throughout, and are intended to encompass any specific and reproducible interaction between an oligonucleotide and a target nucleic acid, including binding of regions having only partial complementarity.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

The stability of a nucleic acid duplex is measured by the melting temperature, or " $T_m$ ." The  $T_m$  of a particular nucleic acid duplex under specified conditions is the temperature at which on average half of the base pairs have disassociated:

The term "probe" as used herein refers to an oligonucleotide which forms a duplex structure or other complex with a sequence in another nucleic acid, due to complementarity or other means of reproducible attractive interaction, of at least one sequence in the probe with a sequence in the other nucleic acid.

5 "Oligonucleotide primers matching or complementary to a gene sequence" refers to oligonucleotide primers capable of facilitating the template-dependent synthesis of single or double-stranded nucleic acids. Oligonucleotide primers matching or complementary to a gene sequence may be used in PCRs, RT-PCRs and the like. As noted above, an oligonucleotide primer need not be perfectly complementary to a  
10 target or template sequence. A primer need only have a sufficient interaction with the template that it can be extended by template-dependent synthesis.

As used herein, the term "poly-histidine tract" or (HIS-tag) refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a nascent protein. A poly-histidine tract of six to ten residues is preferred. The poly-  
15 histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting protein on a nickel-chelate column, or the identification of a protein terminus through the interaction with another molecule (e.g. an antibody reactive with the HIS-tag).

## 20 Descriptions of the Drawings

Figure 1 shows the structure of (A) an amino acid and (B) a peptide.

Figure 2 provides a description of the molecular steps that occur during protein synthesis in a cellular or cell-free system.

Figure 3 shows a structure of (A) a tRNA molecule and (B) approaches  
25 involved in the aminoacylation of tRNAs.

Figure 4 is a schematic representation of the method of detecting nascent proteins using fluorescent marker amino acids.

Figure 5 shows schemes for synthesis and misaminoacylation to tRNA of two different marker amino acids, dansyllysine (scheme 1) and coumarin (scheme 2), with

fluorescent properties suitable for the detection of nascent proteins using gel electrophoresis and UV illumination.

Figure 6(A) shows chemical compounds containing the 2-nitrobenzyl moiety, and Figure 6(B) shows cleavage of substrate from a nitrobenzyl linkage.

5 Figure 7 provides examples of photocleavable markers.

Figure 8(A) shows chemical variations of PCB, and Figure 8(B) depicts possible amino acid linkages.

Figure 9 shows the photolysis of PCB.

10 Figure 10 is a schematic representation of the method for monitoring the production of nascent proteins in a cell-free protein expression systems without separating the proteins.

Figure 11(A) provides examples of non-native amino acids with reporter properties, Figure 11(B) shows participation of a reporter in protein synthesis, and Figure 11(C) shows synthesis of a reporter.

15 Figure 12 shows structural components of photocleavable biotin.

Figure 13 is a schematic representation of the method for introduction of markers at the N-termini of nascent proteins.

Figure 14 provides a description of the method of detection and isolation of marker in nascent proteins.

20 Figure 15 shows the steps in one embodiment for the synthesis of PCB-lysine.

Figure 16 provides an experimental strategy for the misaminoacylation of tRNA.

Figure 17 illustrates dinucleotide synthesis including (i) deoxycytidine protection, (ii) adenosine protection, and (iii) dinucleotide synthesis.

25 Figure 18 depicts aminoacylation of a dinucleotide using marker amino acids.

Figure 19 shows the structure of dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes.

Figure 20 is a photograph of a gel showing the incorporation of various fluorsecent molecules into hemolysin during translation.

30 Figure 21 shows the incorporation of BODIPY-FL into various proteins. Figure

21A shows the results visualized using laser based Molecular Dynamics FluorImager 595, while Figure 21B shows the results visualized using a UV-transilluminator.

Figure 22A shows a time course of fluorescence labeling. Figure 22B shows the SDS-PAGE results of various aliquotes of the translation mixture, demonstrating the sensitivity of the system.

Figure 23A is a bar graph showing gel-free quantitation of an N-terminal marker introduced into a nascent protein in accordance with the method of the present invention. Figure 23B is a bar graph showing gel-free quantitation of an C-terminal marker of a nascent protein quantitated in accordance with the method of the present invention.

Figure 24 shows gel results for protease treated and untreated protein.

Figure 25 shows gel results for protein treated with RBCs and untreated protein.

Figure 26A is a gel showing the incorporation of various fluorescent molecules into  $\alpha$ -hemolysin in *E. coli* translation system using misaminoacylated lysyl-tRNA<sup>lys</sup>. Figure 26B shows the incorporation of various fluorescent molecules into luciferase in a TnT wheat germ system using misaminoacylated lysyl-tRNA<sup>lys</sup>.

Figure 27 shows gel results of *in vitro* translation of  $\alpha$ -HL carried out in the presence of various fluorescent-tRNAs, including atRNA-coumarin derivative.

Figures 28A and 28B show mobility shift results by capillary electrophoresis.

Figure 29 are gel results of *in vitro* translation results wherein three markers were introduced into a nascent protein.

Figure 30 shows Western blot analysis of *in vitro* translated triple-epitope-tagged wild-type p53 (RT-PCR derived DNA). Figure 30A shows the total protein staining. Figure 30B presents the Western blot analysis.

Figure 31 shows the results for a gel-based PTT of APC Exon 15, segment 2.

Figure 32 shows the detection of *in vitro* translated BODIPY-labeled proteins by Western blotting. Figure 32A shows the results by fluorescence imaging and Figure 32B shows the results by Western blotting.

Figure 33 shows three bar graphs representing the results of a gel-free



chemiluminescent protein truncation assay of p53 and APC. Figure 33A shows the results for p53 produced by *in vitro* translation, where the product is captured in a 96-well ELISA plate format using a mouse monoclonal antibody directed against the N-terminal FLAG epitope. Figure 33B shows the results for p53 produced by *in vitro* translation, where the product is captured in a 96-well ELISA plate format using a nickel chelate plate. Figure 33C shows the results for APC produced by *in vitro* translation, where the product is captured on nickel metal chelate 96-well ELISA plates. All WT N- and C-terminal signals as well as mutant N-terminal signals were normalized to 100%.

Figure 34 is a gel showing immunoprecipitation results for p53 and Hemolysin.

Figure 35 schematically shows a synthesis scheme for the preparation of BODIPY-Val-pdCpA.

Figure 36 schematically shows a synthesis scheme for the attachment of the BODIPY-Val-pdCpA conjugate of Figure 35 to tRNA.

Figure 37 shows the result of translation reactions using the charged tRNA of Figure 36.

Figure 38 schematically shows one embodiment of a drug/proteome screening assay.

Figure 39 schematically shows one embodiment of a drug/proteome screening assay.

Figure 40 is a bar graph showing the results of a drug/proteome screening assay.

### Description of the Invention

As embodied and described herein, the present invention comprises methods for the non-radioactive labeling and detection of the products of new or nascent protein synthesis, and methods for the isolation of these nascent proteins from preexisting proteins in a cellular or cell-free translation system. As radioactive labels are not used, there are no special measures which must be taken to dispose of waste materials. There is also no radioactivity danger or risk which would prevent further utilization of

the translation product as occurs when using radioactive labels and the resulting protein product may be used directly or further purified. In addition, no prior knowledge of the protein sequence or structure is required which would involve, for example, unique suppressor tRNAs. Further, the sequence of the gene or mRNA need not be determined. Consequently, the existence of non-sense codons or any specific codons in the coding region of the mRNA is not necessary. Any tRNA can be used, including specific tRNAs for directed labeling, but such specificity is not required. Unlike post-translational labeling, nascent proteins are labeled with specificity and without being subjected to post-translational modifications which may effect protein structure or function.

One embodiment of the invention is directed to a method for labeling nascent proteins synthesized in a translation system. These proteins are labeled while being synthesized with detectable markers which are incorporated into the peptide chain. Markers which are aminoacylated to tRNA molecules, may comprise native amino acids, non-native amino acids, amino acid analogs or derivatives, or chemical moieties. These markers are introduced into nascent proteins from the resulting misaminoacylated tRNAs during the translation process. Aminoacylation is the process whereby a tRNA molecule becomes charged. When this process occurs *in vivo*, it is referred to as natural aminoacylation and the resulting product is an aminoacylated tRNA charged with a native amino acid. When this process occurs through artificial means, it is called misaminoacylation and a tRNA charged with anything but a native amino acid molecule is referred to as a misaminoacylated tRNA.

According to the present method, misaminoacylated tRNAs are introduced into a cellular or cell-free protein synthesizing system, the translation system, where they function in protein synthesis to incorporate detectable marker in place of a native amino acid in the growing peptide chain. The translation system comprises macromolecules including RNA and enzymes, translation, initiation and elongation factors, and chemical reagents. RNA of the system is required in three molecular forms, ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA). mRNA carries the genetic instructions for building a peptide encoded within its codon

sequence. tRNAs contain specific anti-codons which decode the mRNA and individually carry amino acids into position along the growing peptide chain. Ribosomes, complexes of rRNA and protein, provide a dynamic structural framework on which the translation process, including translocation, can proceed. Within the cell,  
5 individualized aminoacyl tRNA synthetases bind specific amino acids to tRNA molecules carrying the matching anti-codon creating aminoacylated or charged tRNAs by the process of aminoacylation. The process of translation including the aminoacylation or charging of a tRNA molecule is described in *Molecular Cell Biology* (J. Darnell et al. editors, Scientific American Books, N.Y., N.Y. 1991), which  
10 is hereby specifically incorporated by reference. Aminoacylation may be natural or by artificial means utilizing native amino acids, non-native amino acid, amino acid analogs or derivatives, or other molecules such as detectable chemicals or coupling agents. The resulting misaminoacylated tRNA comprises a native amino acid coupled with a chemical moiety, non-native amino acid, amino acid derivative or analog, or  
15 other detectable chemicals. These misaminoacylated tRNAs incorporate their markers into the growing peptide chain during translation forming labeled nascent proteins which can be detected and isolated by the presence or absence of the marker.

Any proteins that can be expressed by translation in a cellular or cell-free translation system may be nascent proteins and consequently, labeled, detected and  
20 isolated by the methods of the invention. Examples of such proteins include enzymes such as proteolytic proteins, cytokines, hormones, immunogenic proteins, carbohydrate or lipid binding proteins, nucleic acid binding proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations. These methods are well adapted for the detection of products of recombinant genes and gene fusion  
25 products because recombinant vectors carrying such genes generally carry strong promoters which transcribe mRNAs at fairly high levels. These mRNAs are easily translated in a translation system.

Translation systems may be cellular or cell-free, and may be prokaryotic or eukaryotic. Cellular translation systems include whole cell preparations such as  
30 permeabilized cells or cell cultures wherein a desired nucleic acid sequence can be

transcribed to mRNA and the mRNA translated.

Cell-free translation systems are commercially available and many different types and systems are well-known. Examples of cell-free systems include prokaryotic lysates such as *Escherichia coli* lysates, and eukaryotic lysates such as wheat germ  
5 extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates and human cell lysates. Eukaryotic extracts or lysates may be preferred when the resulting protein is glycosylated, phosphorylated or otherwise modified because many such modifications are only possible in eukaryotic systems. Some of these extracts and  
10 lysates are available commercially (Promega; Madison, WI; Stratagene; La Jolla, CA; Amersham; Arlington Heights, IL; GIBCO/BRL; Grand Island, NY). Membranous extracts, such as the canine pancreatic extracts containing microsomal membranes, are also available which are useful for translating secretory proteins. Mixtures of purified translation factors have also been used successfully to translate mRNA into protein as well as combinations of lysates or lysates supplemented with purified translation  
15 factors such as initiation factor-1 (IF-1), IF-2, IF-3 ( $\alpha$  or  $\beta$ ), elongation factor T (EF-Tu), or termination factors.

Cell-free systems may also be coupled transcription/translation systems wherein DNA is introduced to the system, transcribed into mRNA and the mRNA translated as described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al. editors,  
20 Wiley Interscience, 1993), which is hereby specifically incorporated by reference. RNA transcribed in eukaryotic transcription system may be in the form of heteronuclear RNA (hnRNA) or 5'-end caps (7-methyl guanosine) and 3'-end poly A tailed mature mRNA, which can be an advantage in certain translation systems. For example, capped mRNAs are translated with high efficiency in the reticulocyte lysate  
25 system.

tRNA molecules chosen for misaminoacylation with marker do not necessarily possess any special properties other than the ability to function in the protein synthesis system. Due to the universality of the protein translation system in living systems, a large number of tRNAs can be used with both cellular and cell-free reaction mixtures.  
30 Specific tRNA molecules which recognize unique codons, such as nonsense or amber

codons (UAG), are not required.

Site-directed incorporation of the nonnative analogs into the protein during translation is also not required. Incorporation of markers can occur anywhere in the polypeptide and can also occur at multiple locations. This eliminates the need for prior information about the genetic sequence of the translated mRNA or the need for modifying this genetic sequence.

In some cases, it may be desirable to preserve the functional properties of the nascent protein. A subset of tRNAs which will incorporate markers at sites which do not interfere with protein function or structure can be chosen. Amino acids at the amino or carboxyl terminus of a polypeptide do not alter significantly the function or structure. tRNA molecules which recognize the universal codon for the initiation of protein translation (AUG), when misaminoacylated with marker, will place marker at the amino terminus. Prokaryotic protein synthesizing systems utilize initiator tRNA<sup>fMet</sup> molecules and eukaryotic systems initiator tRNA<sup>Met</sup> molecules. In either system, the initiator tRNA molecules are aminoacylated with markers which may be non-native amino acids or amino acid analogs or derivatives that possess marker, reporter or affinity properties. The resulting nascent proteins will be exclusively labeled at their amino terminus, although markers placed internally do not necessarily destroy structural or functional aspects of a protein. For example, a tRNA<sup>LYS</sup> may be misaminoacylated with the amino acid derivative dansyllysine which does not interfere with protein function or structure. In addition, using limiting amounts of misaminoacylated tRNAs, it is possible to detect and isolate nascent proteins having only a very small fraction labeled with marker which can be very useful for isolating proteins when the effects of large quantities of marker would be detrimental or are unknown.

tRNAs molecules used for aminoacylation are commercially available from a number of sources and can be prepared using well-known methods from sources including *Escherichia coli*, yeast, calf liver and wheat germ cells (Sigma Chemical; St. Louis, MO; Promega; Madison, WI; Boehringer Mannheim Biochemicals; Indianapolis, IN). Their isolation and purification mainly involves cell-lysis; phenol extraction...

followed by chromatography on DEAE-cellulose. Amino-acid specific tRNA, for example tRNA<sup>fMet</sup>, can be isolated by expression from cloned genes and overexpressed in host cells and separated from total tRNA by techniques such as preparative polyacrylamide gel electrophoresis followed by band excision and elution in high yield and purity (Seong and RajBhandary, Proc. Natl. Acad. Sci. USA 84:334-338, 1987). Run-off transcription allows for the production of any specific tRNA in high purity, but its applications can be limited due to lack of post-transcriptional modifications (Bruce and Uhlenbeck, Biochemistry 21:3921, 1982).

Misaminoacylated tRNAs are introduced into the cellular-or cell-free protein synthesis system. In the cell-free protein synthesis system, the reaction mixture contains all the cellular components necessary to support protein synthesis including ribosomes, tRNA, rRNA, spermidine and physiological ions such as magnesium and potassium at appropriate concentrations and an appropriate pH. Reaction mixtures can be normally derived from a number of different sources including wheat germ, *E. coli* (S-30), red blood cells (reticulocyte lysate,) and oocytes, and once created can be stored as aliquots at about +4°C to -70°C. The method of preparing such reaction mixtures is described by J.M. Pratt (*Transcription and Translation*, B.D. Hames and S.J. Higgins, Editors, p. 209, IRL Press, Oxford, 1984) which is hereby incorporated by reference. Many different translation systems are commercially available from a number of manufacturers.

The misaminoacylated tRNA is added directly to the reaction mixture as a solution of predetermined volume and concentration. This can be done directly prior to storing the reaction mixture at -70°C in which case the entire mixture is thawed prior to initiation of protein synthesis or prior to the initiation of protein synthesis. Efficient incorporation of markers into nascent proteins is sensitive to the final pH and magnesium ion concentration. Reaction mixtures are normally about pH 6.8 and contain a magnesium ion concentration of about 3 mM. These conditions impart stability to the base-labile aminoacyl linkage of the misaminoacylated tRNA. Aminoacylated tRNAs are available in sufficient quantities from the translation extract. Misaminoacylated tRNAs charged with markers are added at between about 1.0 µg /ml

to about 1.0 mg/ml, preferably at between about 10 µg/ml to about 500 µg/ml, and more preferably at about 150 µg/ml.

Initiation of protein synthesis occurs upon addition of a quantity of mRNA or DNA to the reaction mixture containing the misaminoacylated tRNAs. mRNA  
5 molecules may be prepared or obtained from recombinant sources, or purified from other cells by procedure such as poly-dT chromatography. One method of assuring that the proper ratio of the reaction mixture components is to use predetermined volumes that are stored in convenient containers such as vials or standard microcentrifuge tubes. For example, DNA and/or mRNA coding for the nascent  
10 proteins and the misaminoacylated tRNA solution are premixed in proper amounts and stored separately in tubes. Tubes are mixed when needed to initiate protein synthesis.

Translations in cell-free systems generally require incubation of the ingredients for a period of time. Incubation times range from about 5 minutes to many hours, but is preferably between about thirty minutes to about five hours and more preferably  
15 between about one to about three hours. Incubation may also be performed in a continuous manner whereby reagents are flowed into the system and nascent proteins removed or left to accumulate using a continuous flow system (A.S. Spirin et al., Sci. 242:1162-64, 1988). This process may be desirable for large scale production of nascent proteins. Incubations may also be performed using a dialysis system where  
20 consumable reagents are available for the translation system in an outer reservoir which is separated from larger components of the translation system by a dialysis membrane [Kim, D., and Choi, C. (1996) *Biotechnol Prog* 12, 645-649]. Incubation times vary significantly with the volume of the translation mix and the temperature of the incubation. Incubation temperatures can be between about 4°C to about 60°C, and  
25 are preferably between about 15°C to about 50°C, and more preferably between about 25°C to about 45°C and even more preferably at about 25°C or about 37°C. Certain markers may be sensitive to temperature fluctuations and in such cases, it is preferable to conduct those incubations in the non-sensitive ranges. Translation mixes will  
30 typically comprise buffers such as Tris-HCl, Hepes or another suitable buffering agent to maintain the pH of the solution between about 6 to 8, and preferably at about 7.

Again, certain markers may be pH sensitive and in such cases, it is preferable to conduct incubations outside of the sensitive ranges for the marker. Translation efficiency may not be optimal, but marker utility will be enhanced. Other reagents which may be in the translation system include dithiothreitol (DTT) or 2-mercaptoethanol as reducing agents, RNasin to inhibit RNA breakdown, and nucleoside triphosphates or creatine phosphate and creatine kinase to provide chemical energy for the translation process.

In cellular protein synthesis, it is necessary to introduce misaminoacylated tRNAs or markers into intact cells, cell organelles, cell envelopes and other discrete volumes bounded by an intact biological membrane, which contain a protein synthesizing system. This can be accomplished through a variety of methods that have been previously established such as sealing the tRNA solution into liposomes or vesicles which have the characteristic that they can be induced to fuse with cells. Fusion introduces the liposome or vesicle interior solution containing the tRNA into the cell. Alternatively, some cells will actively incorporate liposomes into their interior cytoplasm through phagocytosis. The tRNA solution could also be introduced through the process of cationic detergent mediated lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-17, 1987), or injected into large cells such as oocytes. Injection may be through direct perfusion with micropipettes or through the method of electroporation.

Alternatively, cells can be permeabilized by incubation for a short period of time in a solution containing low concentrations of detergents in a hypotonic media. Useful detergents include Nonidet-P 40 (NP40), Triton X-100 (TX-100) or deoxycholate at concentrations of about 0.01 nM to 1.0 mM, preferably between about 0.1  $\mu$ M to about 0.01 mM, and more preferably about 1  $\mu$ M. Permeabilized cells allow marker to pass through cellular membranes unaltered and be incorporated into nascent proteins by host cell enzymes. Such systems can be formed from intact cells in culture such as bacterial cells, primary cells, immortalized cell lines, human cells or mixed cell populations. These cells may, for example, be transfected with an appropriate vector containing the gene of interest, under the control of a strong and



possibly regulated promoter. Messages are expressed from these vectors and subsequently translated within cells. Intact misaminoacylated tRNA molecules, already charged with a non-radioactive marker could be introduced to cells and incorporated into translated product.

5 One example of the use of misaminoacylation to detect nascent protein is schematically represented in Figure 4. A tRNA molecule is misaminoacylated with the marker which is highly fluorescent when excited with UV (ultraviolet) radiation. The misaminoacylated tRNA is then introduced into a cell-free protein synthesis extract and the nascent proteins containing the marker analog produced. Proteins in the cell-free  
10 extract are separated by polyacrylamide gel electrophoresis (PAGE). The resulting gel contains bands which correspond to all of the proteins present in the cell-free extract. The nascent protein is identified upon UV illumination of the gel by detection of fluorescence from the band corresponding to proteins containing marker. Detection can be through visible observation or by other conventional means of fluorescence  
15 detection.

The misaminoacylated tRNA can be formed by natural aminoacylation using cellular enzymes or misaminoacylation such as chemical misaminoacylation. One type of chemical misaminoacylation involves truncation of the tRNA molecule to permit attachment of the marker or marker precursor. For example, successive treatments  
20 with periodate plus lysine, pH 8.0, and alkaline phosphatase removes 3'-terminal residues of any tRNA molecule generating tRNA-OH-3' with a mononucleotide or dinucleotide deletion from the 3'-terminus (Neu and Heppel, J. Biol. Chem. 239:2927-34, 1964). Alternatively, tRNA molecules may be genetically manipulated to delete specific portions of the tRNA gene. The resulting gene is transcribed producing  
25 truncated tRNA molecules (Sampson and Uhlenbeck, Proc. Natl. Acad. Sci. USA 85:1033-37, 1988). Next, a dinucleotide is chemically linked to a modified amino acid or other marker by, for example, acylation. Using this procedure, markers can be synthesized and acylated to dinucleotides in high yield (Hudson, J. Org. Chem. 53:617-624, 1988; Happ et al., J. Org. Chem. 52:5387-91, 1987). These modified groups are  
30 bound together and linked via the dinucleotide to the truncated tRNA molecules in a

process referred to as ligase coupling (Figure 3B).

A different bond is involved in misaminoacylation (Figure 3B, link B) than the bond involved with activation of tRNA by aminoacyl tRNA synthetase (Figure 3B, link A). As T4 RNA ligase does not recognize the acyl substituent, tRNA molecules  
5 can be readily misaminoacylated with few chemical complications or side reactions (link B, Figure 3B) (T.G. Heckler et al., Biochemistry 23:1468-73, 1984; and T.G. Heckler et al., Tetrahedron 40:87-94, 1984). This process is insensitive to the nature of the attached amino acid and allows for misaminoacylation using a variety of non-native amino acids. In contrast, purely enzymatic aminoacylation (link A) is highly  
10 sensitive and specific for the structures of substrate tRNA and amino acids.

Markers are basically molecules which will be recognized by the enzymes of the translation process and transferred from a charged tRNA into a growing peptide chain. To be useful, markers must also possess certain physical and physio-chemical properties. Therefore, there are multiple criteria which can be used to identify a useful  
15 marker. First, a marker must be suitable for incorporation into a growing peptide chain. This may be determined by the presence of chemical groups which will participate in peptide bond formation. Second, markers should be attachable to a tRNA molecule. Attachment is a covalent interaction between the 3'-terminus of the tRNA molecule and the carboxy group of the marker or a linking group attached to the  
20 marker and to a truncated tRNA molecule. Linking groups may be nucleotides, short oligonucleotides or other similar molecules and are preferably dinucleotides and more preferably the dinucleotide CA. Third, markers should have one or more physical properties that facilitate detection and possibly isolation of nascent proteins. Useful physical properties include a characteristic electromagnetic spectral property such as  
25 emission or absorbance, magnetism, electron spin resonance, electrical capacitance, dielectric constant or electrical conductivity.

Useful markers are native amino acids coupled with a detectable label, detectable non-native amino acids, detectable amino acid analogs and detectable amino acid derivatives. Labels and other detectable moieties may be ferromagnetic,  
30 paramagnetic, diamagnetic, luminescent, electrochemiluminescent, fluorescent,

phosphorescent, chromatic or have a distinctive mass. Fluorescent moieties which are useful as markers include dansyl fluorophores, coumarins and coumarin derivatives, fluorescent acridinium moieties and benzopyrene based fluorophores. Preferably, the fluorescent marker has a high quantum yield of fluorescence at a wavelength different from native amino acids and more preferably has high quantum yield of fluorescence can be excited in both the UV and visible portion of the spectrum. Upon excitation at a preselected wavelength, the marker is detectable at low concentrations either visually or using conventional fluorescence detection methods. Electrochemiluminescent markers such as ruthenium chelates and its derivatives or nitroxide amino acids and their derivatives are preferred when extreme sensitivity is desired (J. DiCesare et al., BioTechniques 15:152-59, 1993). These markers are detectable at the femtomolar ranges and below.

In addition to fluorescent markers, a variety of markers possessing other specific physical properties can be used to detect nascent protein production. In general, these properties are based on the interaction and response of the marker to electromagnetic fields and radiation and include absorption in the UV, visible and infrared regions of the electromagnetic spectrum, presence of chromophores which are Raman active, and can be further enhanced by resonance Raman spectroscopy, electron spin resonance activity and nuclear magnetic resonances and use of a mass spectrometer to detect presence of a marker with a specific molecular mass. These electromagnetic spectroscopic properties are preferably not possessed by native amino acids or are readily distinguishable from the properties of native amino acids. For example, the amino acid tryptophan absorbs near 290 nm, and has fluorescent emission near 340 nm when excited with light near 290 nm. Thus, tryptophan analogs with absorption and/or fluorescence properties that are sufficiently different from tryptophan can be used to facilitate their detection in proteins.

Many different modified amino acids which can be used as markers are commercially available (Sigma Chemical; St. Louis, MO; Molecular Probes; Eugene, OR). One such marker is Nε-dansyllysine created by the misaminoacylation of a dansyl fluorophore to a tRNA molecule (Figure 5; scheme 1). The α-amino group of

Nε-dansyllysine is first blocked with NVOC (ortho-nitro veratryl oxycarbonyl chloride) and the carboxyl group activated with cyanomethyl ester. Misaminoacylation is performed as described. The misaminoacylated tRNA molecules are then introduced into the protein synthesis system, whereupon the dansyllysine is incorporated directly into the newly synthesized proteins.

Another such marker is a fluorescent amino acid analog based on the highly fluorescent molecule coumarin (Figure 5; scheme 2). This fluorophore has a much higher fluorescence quantum yield than dansyl chloride and can facilitate detection of much lower levels of nascent protein. In addition, this coumarin derivative has a structure similar to the native amino acid tryptophan. These structural similarities are useful where maintenance of the nascent proteins' native structure or function are important or desired. Coumarin is synthesized as depicted in Figure 5 (scheme 2). Acetamidomalonate is alkylated with a slight excess of 4-bromomethyl coumarin (Aldrich Chemicals; Milwaukee; WI) in the presence of sodium ethoxide followed by acid hydrolysis. The corresponding amino acid as a hydrochloride salt that can be converted to the free amino acid analog.

The coumarin derivative can be used most advantageously if it misamino-acylates the tryptophan-tRNA, either enzymatically or chemically. When introduced in the form of the misaminoacylated tryptophan-tRNA, the coumarin amino acid will be incorporated only into tryptophan positions. By controlling the concentration of misaminoacylated tRNAs or free coumarin derivatives in the cell-free synthesis system, the number of coumarin amino acids incorporated into the nascent protein can also be controlled. This procedure can be utilized to control the amount of most any markers in nascent proteins.

Markers can be chemically synthesized from a native amino acid and a molecule with marker properties which cannot normally function as an amino acid. For example a highly fluorescent molecule can be chemically linked to a native amino acid group. The chemical modification can occur on the amino acid side-chain, leaving the carboxyl and amino functionalities free to participate in a polypeptide bond formation. Highly fluorescent molecules (e.g. dansyl chloride) can be linked to the

nucleophilic side chains of a variety of amino acids including lysine, arginine, tyrosine, cysteine, histidine, etc., mainly as a sulfonamide for amino groups or sulfate bonds to yield fluorescent derivatives. Such derivatization leaves the ability to form peptide bond intact, allowing the normal incorporation of dansyllysine into a protein.

A number of factors determine the usefulness of a marker which is to be incorporated into nascent proteins through misaminoacylated tRNAs. These include the ability to incorporate the marker group into the protein through the use of a misaminoacylated tRNA in a cell-free or cellular protein synthesis system and the intrinsic detectability of the marker once it is incorporated into the nascent protein. In general, markers with superior properties will allow shorter incubation times and require smaller samples for the accurate detection of the nascent proteins. These factors directly influence the usefulness of the methods described. In the case of fluorescent markers used for the incorporation into nascent proteins, favorable properties can be but are not limited to, small size, high quantum yield of fluorescence, and stability to prolonged light exposure (bleach resistance).

Even with knowledge of the above factors, the optimum conditions to incorporate a specific marker into a protein using a specific cell-free or cellular translation system is difficult to predict *a priori* since it depends on the detailed interaction of the marker group with components of the protein translational synthesis system including the tRNA, initiation or elongation factors and components of the ribosome. While it is generally expected that markers with smaller sizes can be accommodated more readily into the ribosome, the exact shape of the molecule and its specific interactions in the ribosomal binding site will be the most important determinant. For this reason, it is possible that some markers which are larger in size can be more readily incorporated into nascent proteins compared to smaller markers. For example, such factors are very difficult to predict using known methods of molecular modeling.

One group of fluorophores with members possessing several favorable properties (including favorable interactions with components of the protein translational synthesis system) is the group derived from dipyrrometheneboron



530 nm. U.S. Pat. No. 4,774,339 to Haugland et al. (1988) ('339 patent) (hereby incorporated by reference) describes 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (dipyrrrometheneboron difluoride) dyes including hydrogen, halogen, alkyl, cycloalkyl, aryl, arylalkyl, acyl, and sulfo-substituted derivatives that contain reactive groups  
5 suitable for conjugation to biomolecules, that have good photostability, and which have fluorescein-like spectra. As described in the '339 patent, and by Pavlopoulos, et al., [Laser action from a tetramethylpyrromethene-BF<sub>3</sub> complex, APP. OPTICS 27, 4998 (1988)], the emission of the alkyl derivatives of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene fluorescent dyes clearly overlaps that of fluorescein. The overlap allows the  
10 alkyl derivatives of dipyrrrometheneboron difluoride to be used with the same optical equipment as used with fluorescein-based dyes without modification of the excitation sources or optical filters. Similarly, aryl/heteroaryl substituents in the dipyrrrometheneboron difluoride cause the maximum of absorbance/emission to shift into longer wavelengths (See U.S. Pat. 5,451,663 hereby incorporated by reference).

15 A variety of BODIPY molecules are commercially available in an amine reactive form which can be used to derivitize aminoacylated tRNAs to yield a misaminoacylated tRNA with a BODIPY marker moiety. One example of a compound from this family which exhibits superior properties for incorporation of a detectable marker into nascent proteins is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-  
20 diaza-s-indacene (BODIPY-FL). When the sulfonated N-hydroxysuccinimide (NHS) derivative of BODIPY-FL is used to misaminoacylate an *E. coli* initiator tRNA<sup>fmet</sup>, the nascent protein produced can be easily detected on polyacrylamide gels after electrophoresis using a standard UV-transilluminator and photographic or CCD  
25 imaging system. This can be accomplished by using purified tRNA<sup>fmet</sup> which is first aminoacylated with methionine and then the  $\alpha$ -amino group of methionine is specifically modified using N-hydroxysuccinimide BODIPY. Before the modification reaction, the tRNA<sup>fmet</sup> is charged maximally (>90%) and confirmed by using <sup>35</sup>S-methionine and acid-urea gels [Varshney, U., Lee, C. P., and RajBhandary, U. L. 1991. Direct analysis of aminoacylation levels of tRNA *in vitro*. *J. Biol. Chem.*  
30 266:24712-24718] .

Less than 10 nanoliters of a commercially available *E.coli* extract (*E. coli* T7 translation system, Promega, Madison, WI) are needed for analysis corresponding to less than 1 ng of synthesized protein. Incubation times required to produce detectable protein is approximately 1 hour but can be as little as 5 minutes (or less). BODIPY-FL can also be detected with higher sensitivity using commercially available fluorescent scanners with 488 nm excitation and emission measurement above 520 nm. Similar tests using other commercially available dyes including NBD (7-Nitrobenz-2-Oxa-1,3-Diazole) , and Pyrene-PyMPO show approximately an order of magnitude reduction in fluorescence making them more difficult to detect using standard laboratory equipment such as a UV-transilluminator or fluorescent scanner. It has previously been shown that fluorescent markers such as 3-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-2,3-diaminopropionic acid (NBD-DAP) and coumarin could be incorporated into proteins using misaminoacylated tRNAs. However, detection of nascent proteins containing these markers was only demonstrated using highly sensitive instrumentation such a fluorescent spectrometer or a microspectrofluorimeter and often require indirect methods such as the use of fluorescence resonance energy transfer (FRET) (Turcatti, G., Nemeth, K., Edgerton, M. D., Meseth, U., Talabot, F., Peitsch, M., Knowles, J., Vogel, H., and Chollet, A. (1996) *J Biol Chem* 271(33), 19991-8; Kudlicki, W., Odom, O. W., Kramer, G., and Hardesty, B. (1994) *J Mol Biol* 244(3), 319-31). Such instruments are generally not available for routine use in a molecular biology laboratory and only with special adaptation can be equipped for measurement of fluorescent bands on a gel.

An additional advantage of BODIPY-FL as a marker is the availability of monoclonal antibodies directed against it which can be used to affinity purify nascent proteins containing said marker. One example of such a monoclonal antibody is anti-BODIPY-FL antibody (Cat# A-5770, Molecular Probes, Eugene, OR). This combined with the ability incorporate BODIPY-FL into nascent proteins with high efficiency relative to other commercially available markers using misaminoacylated tRNAs facilitates more efficient isolation of the nascent protein. These antibodies against BODIPY-FL can be used for quantitation of incorporation of the BODIPY into the



nascent protein.

A marker can also be modified after the tRNA molecule is aminoacylated or misaminoacylated using chemical reactions which specifically modify the marker without significantly altering the functional activity of the aminoacylated tRNA. These types of post-aminoacylation modifications may facilitate detection, isolation or purification, and can sometimes be used where the modification allow the nascent protein to attain a native or more functional configuration.

Fluorescent and other markers have detectable electromagnetic spectral properties that can be detected by spectrometers and distinguished from the electromagnetic spectral properties of native amino acids. Spectrometers which are most useful include fluorescence, Raman, absorption, electron spin resonance, visible, infrared and ultraviolet spectrometers. Other markers, such as markers with distinct electrical properties can be detected by an apparatus such as an ammeter, voltmeter or other spectrometer. Physical properties of markers which relate to the distinctive interaction of the marker with an electromagnetic field is readily detectable using instruments such as fluorescence, Raman, absorption, electron spin resonance spectrometers. Markers may also undergo a chemical, biochemical, electrochemical or photochemical reaction such as a color change in response to external forces or agents such as an electromagnetic field or reactant molecules which allows its detection.

One class of fluorescent markers contemplated by the present invention is the class of small peptides that can specifically bind to molecules which, upon binding, are detectable. One example of this approach is the peptide having the sequence of WEAAAREACCRECCARA (SEQ ID NO: 4). This sequence (which contains four cysteine residues) allows the peptide to specifically bind the non-fluorescent dye molecule 4', 5'-bis(1,3,2-dithioarsolan-2-yl) fluorescein (FLASH, which stands for fluorescein arsenic helix binder). This dye has the interesting property that, upon binding, it becomes fluorescent. In other words, fluorescence is observed only when this specific peptide sequence is present in the nascent protein. So by putting the peptide sequence at the N- or C-terminal, one can easily monitor the amount of protein synthesized. This peptide sequence can be introduced by designing the nucleic acid

primers such that they carry a region encoding the peptide sequence.

Regardless of which class of fluorescent compounds is used, detection normally first involves physical separation of the nascent proteins from other biomolecules present in the cellular or cell-free protein synthesis system. Protein separation can be performed using, for example, gel electrophoresis or column chromatography and can be further facilitated with affinity markers which uniquely bind acceptor groups. Detection of a marker containing a fluorophore by gel electrophoresis can be accomplished using conventional fluorescence detection methods.

After protein synthesis in a cell-free system, the reaction mixture, which contains all of the biomolecules necessary for protein synthesis as well as nascent proteins, is loaded onto a gel which may be composed of polyacrylamide or agarose (R.C. Allen et al., *Gel Electrophoresis and Isoelectric Focusing of Proteins*, Walter de Gruyter, New York 1984). This mixture also contains the misaminoacylated tRNAs bearing the marker as well as uncharged tRNAs. Subsequent to loading the reaction mixture, a voltage is applied which spatially separates the proteins on the gel in the direction of the applied electric field. The proteins separate and appear as a set of discrete or overlapping bands which can be visualized using a pre- or post-gel staining technique such as Coomassie blue staining. The migration of the protein band on the gel is a function of the molecular weight of the protein with increasing distance from the loading position being a function of decreasing molecular weight. Bands on the gel which contain nascent proteins will exhibit fluorescence when excited at a suitable wavelength. These bands can be detected visually, photographically or spectroscopically and, if desired, the nascent proteins purified from gel sections.

For example, if BODIPY-FL is used as a marker, nascent proteins will fluoresce at 510 nm when excited by UV illumination. This fluorescence can be detected visually by simply using a standard hand-held UV illuminator or a transilluminator. Approximately 10 nanograms (ng) of the protein alpha-hemolysin is detectable using this method. Also useful are electronic imaging devices which can rapidly screen and identify very low concentrations of markers such as a fluorescent scanner based on a low-temperature CCD imager. In this case as low as 0.3 ng of

protein can be detected.

The molecular weight and quantity of the nascent protein can be determined by comparison of its band-position on the gel with a set of bands of proteins of predetermined molecular weight which are fluorescently labeled. For example, a nascent protein of molecular weight 25,000 could be determined because of its relative position on the gel relative to a calibration gel containing the commercially available standard marker proteins of known quantities and with known molecular weights (bovine serum albumin, 66 kD; porcine heart fumarase, 48.5 kD; carbonic anhydrase, 29 kD,  $\beta$ -lactoglobulin, 18.4 kD;  $\alpha$ -lactoglobulin, 14.2 kD; Sigma Chemical; St. Louis, MO).

Calibration proteins may also contain a similar markers for convenient detection using the same method as the gel bearing the nascent protein. This can be accomplished in many cases by directly reacting the calibration proteins with a molecule similar to the marker. For example, the calibration proteins can be modified with dansyl chloride so as to obtain their fluorescent derivatives (R.E. Stephens, Anal. Biochem. 65, 369-79, 1975). Alternatively, the proteins could be labeled with an NHS derivative of BODIPY-FL. These fluorescent proteins can be analyzed using PAGE. Combined detection of these fluorescent calibration proteins along with that of nascent protein which contains fluorescent marker analog will accurately determine both the molecular weight and quantity of the nascent protein synthesized. If necessary, the amounts of marker within each calibration and nascent protein can be determined to provide an accurate quantitation. Proteins with predetermined levels of fluorescent markers can be used advantageously to provide for quantitation of the gel bearing the nascent protein. This could be accomplished by genetically engineering a calibration protein so that it contains a specific reactive residue such as cysteine so that only one fluorescent dye will be attached per protein.

Other methods of protein separation are also useful for detection and subsequent isolation and purification of nascent proteins containing markers. For example, proteins can be separated using capillary electrophoresis, isoelectric focusing, low pressure chromatography and high-performance or fast-pressure liquid

chromatography (HPLC or FPLC). In these cases, the individual proteins are separated into fractions which can be individually analyzed by fluorescent detectors at the emission wavelengths of the markers. Alternatively, on-line fluorescence detection can be used to detect nascent proteins as they emerge from the column fractionation system. A graph of fluorescence as a function of retention time provides information on both the quantity and purity of nascent proteins produced.

Another embodiment of the invention is directed to a method for labeling, detecting and, if desired, isolating and purifying nascent proteins, as described above, containing cleavable markers. Cleavable markers comprise a chemical structure which is sensitive to external effects such as physical or enzymatic treatments, chemical or thermal treatments, electromagnetic radiation such as gamma rays, x-rays, ultraviolet light, visible light, infrared light, microwaves, radio waves or electric fields. The marker is aminoacylated to tRNA molecules as before using conventional technology or misaminoacylated and added to a translation system. After incubation and production of nascent proteins, marker can be cleaved by the application of specified treatments and nascent proteins detected. Alternatively, nascent proteins may also be detected and isolated by the presence or absence of the cleaved marker or the chemical moiety removed from the marker.

One example of a cleavable marker is a photocleavable marker such as chemical compounds which contain the 2-nitrobenzyl moiety (Figure 6A). Upon illumination, these aromatic nitro compounds undergo an internal oxidation-reduction reaction (Pillai, Synthesis 1-26, 1980; Patchornik *et al.*, *J. Am. Chem. Soc.* 92:6333-35, 1970). As a result, the nitro group is reduced to a nitroso group and an oxygen is inserted into the benzylic carbon-hydrogen bond at the ortho position. The primary photochemical process is the intramolecular hydrogen abstraction by the excited nitro group. This is followed by an electron-redistribution process to the aci-nitro form which rearranges to the nitroso product. Subsequent thermal reaction leads to the cleavage of substrate from the nitrobenzyl linkage (Figure 6B). Examples of other cleavable markers are shown in Figure 7.

It may sometimes be desirable to create a distance between the substrate and

the cleavable moiety. To accomplish this, cleavable moieties may be separated from substrates by cross-linker arms. Cross-linkers increase substrate access and stabilize the chemical structure, and can be constructed using, for example, long alkyl chains or multiple repeat units of caproyl moieties linked via amide linkages.

5           There are as many methods to synthesize cleavable markers as there are different markers. One example for the synthesis of photocleavable biotins based on nitrobenzyl alcohols involves four major steps. 2-bromo-2'-nitroacetphenone, a precursor of the photoreactive moiety, is first converted into an  $\alpha$ - or  $\omega$ -amino acid like  $\epsilon$ -aminocaprylic acid. The resulting acid and amino functionality of the  
10           photoreactive group is coupled using dicyclohexyl carbodiimide (DCC). The benzoyl carbonyl group of the resulting amide is reduced using sodium borohydride. The resulting derivative of nitrobenzyl alcohol is derivatized to obtain the final component which is able to react with biomolecular substrates, for example by the reaction with phosgene gas, to introduce the chloroformate functionality. The resulting compound is  
15           depicted in Figure 8A along with alternative derivatives of PCB. Possible linkages to amino acids are depicted in Figure 8B.

Cleavable markers can facilitate the isolation of nascent proteins. For example, one type of a cleavable marker is photocleavable biotin coupled to an amino acid. This marker can be incorporated into nascent proteins and the proteins purified by the  
20           specific interaction of biotin with avidin or streptavidin. Upon isolation and subsequent purification, the biotin is removed by application of electromagnetic radiation and nascent proteins utilized in useful applications without the complications of an attached biotin molecule. Other examples of cleavable markers include photocleavable coumarin, photocleavable dansyl, photocleavable dinitrophenyl and  
25           photocleavable coumarin-biotin. Photocleavable markers are cleaved by electromagnetic radiation such as UV light, peptidyl markers are cleaved by enzymatic treatments, and pyrenyl fluorophores linked by disulfide bonds are cleaved by exposure to certain chemical treatments such as thiol reagents.

Cleavage of photocleavable markers is dependent on the structure of the  
30           photoreactive moiety and the wavelength of electromagnetic radiation used for

illumination. Other wavelengths of electromagnetic radiation should not damage the proteins or other chemical moieties. In the case of unsubstituted 2-nitrobenzyl derivatives, the yield of photolysis and recovery of the substrate are significantly decreased by the formation of side products which act as internal light filters and are capable to react with amino groups of the substrate. Typical illumination times vary from 1 to about 24 hours and yields are 1-95%. Radiation sources are placed within about 10 cm of the substrate proteins and set on low power so as to minimize side reactions, if any, which may occur in the nascent proteins. In the case of alpha-substituted 2-nitrobenzyl derivatives (methyl, phenyl, etc.), a considerable increase in rate of photo-removal as well as yield of the released substrate are observed. The introduction of other electron donor groups into phenyl rings of photoreactive moieties increases the yield of substrate. The general reaction for the photolysis of PCB is depicted in Figure 9.

For enzymatic cleavage, markers introduced contain specific bonds which are sensitive to unique enzymes of chemical substances. Introduction of the enzyme or chemical into the protein mixture cleaves the marker from the nascent protein. When the marker is a modified amino acid, this can result in the production of native protein forms. Thermal treatments of, for example, heat sensitive chemical moieties operate in the same fashion. Mild application of thermal energy, such as with microwaves or radiant heat, cleaves the sensitive marker from the protein without producing any significant damage to the nascent proteins.

## **Description of Preferred Embodiments**

### **A. Detection of Mutations**

Detection of mutations is an increasingly important area in clinical diagnosis, including but not limited to the diagnosis of cancer and/or individuals disposed to cancer. The protein truncation test (PTT) is a technique for the detection of nonsense and frameshift mutations which lead to the generation of truncated protein products. Genes associated with Duchenne muscular dystrophy, adenomatous polyposis coli, human mutL homologue and human nutS homologue (both involved in colon cancer),

and BRAC1 (involved in familial breast cancer) can now be screened for mutations in this manner, along with others (see Table 1).

Typically, the PTT technique involves the incorporation of a T7 promoter site, ribosome binding site, and an artificial methionine start site into a PCR product covering the region of the gene to be investigated. The PCR product is then transcribed and translated using either an in vitro rabbit reticulocyte lysate or wheat germ lysate system, to generate a protein corresponding to the region of the gene amplified. The presence of a stop codon in the sequence, generated by a nonsense mutation or a frameshift, will result in the premature termination of protein translation, producing a truncated protein that can be detected by standard gel electrophoresis (e.g. SDS-PAGE) analysis combined with radioactive detection.

There are drawbacks to the technique as currently practiced. One of the most important problems involves the identification of the product of interest. This is made difficult because of nonspecific radiolabeled products. Attempts to address these problems have been made. One approach is to introduce an affinity tag after the start site and before the region encoding the gene of interest. See Rowan and Bodmer, "Introduction of a *myc* Reporter Tag to Improve the Quality of Mutation Detection Using the Protein Truncation Test," *Human Mutation* 9:172 (1997). However, such approaches still have the disadvantage that they rely on electrophoresis.

The present invention contemplates a gel-free truncation test (GFTT), wherein two or three markers are introduced into the nascent protein. The present invention contemplates both pre-natal and post-natal testing to determine predisposition to disease. In a preferred embodiment of the invention, the novel compositions and methods are directed to the detection of frameshift or chain terminating mutations. In order to detect such mutations, a nascent protein is first synthesized in a cell-free or cellular translation system from message RNA or DNA coding for the protein which may contain a possible mutation. The nascent protein is then separated from the cell-free or cellular translation system using an affinity marker located at or close to the

**Table 1. Applications of PTT in Human Molecular Genetics**

Disease References	% Truncating Mutations	Gene
Familial Adenomatous Polyposis	95%	APC
Hereditary desmold disease	100%	APC
Ataxia telangiectasia	90%	ATM
Hereditary Breast and Ovarian Cancer	90%	BRCA1
	90%	BRCA2
Cystic Fibrosis	15%	CFTR
Duchenne Muscular Dystrophy	95%	DMD
Emery-Dreifuss Muscular Dystrophy	80%	EMD
Fanconi anaemia	80%	FAA
Hunter Syndrome	-50%	IDS
Hereditary non-polyposis colorectal cancer	-80%	hMSH2
	-70%	hMLH1
Neurofibromatosis type 1	50%	NF1
Neurofibromatosis type 2	65%	NF2
Polycystic Kidney Disease	95%	PKD1
Rubinstein-Taybi Syndrome	10%	RTS
<i>The percentage of truncating mutations reported which should be detectable using PTT.</i>		





specific protein sequence known as an epitope. An epitope has the property that it selectively interacts with molecules and/or materials containing acceptor groups. There are many epitope sequences reported in the literature including HisX6 (HHHHHH) (SEQ ID NO: 5) described by ClonTech and C-myc (-EQKLISEEDL) (SEQ ID NO:6) described by Roche-BM, Flag (DYKDDDDK) (SEQ ID NO:7) described by Stratagene), SteptTag (WSHPQFEK) (SEQ ID NO:8) described by Sigma-Genosys and HA Tag (YPYDVPDYA) (SEQ ID NO:9) described by Roche-BM.

Once the nascent protein is isolated from the translation system, it is analyzed for presence of the detectable marker incorporated at the N-terminal of the protein. In the case of BODIPY FL, it can be detected by measuring the level of fluorescence using a variety of commercially available instrument such as a Molecular Dynamics Model 595 fluorescence scanner that is equipped with excitation at 488 nm and an emission filter that allows light above 520 nm to be transmitted to the detector.

The protein is then analyzed for the presence of a sequence specific marker located near the C-terminal end of the protein. In normal practice, such a sequence specific marker will consist of a specific sequence of amino acids located near the C-terminal end of the protein which is recognized by a coupling agent. For example, an antibody can be utilized which is directed against an amino acid sequence located at or near C-terminal end of the nascent protein can be utilized. Such antibodies can be labeled with a variety of markers including fluorescent dyes that can be easily detected and enzymes which catalyze detectable reactions that lead to easily detectable substrates. The marker chosen should have a different detectable property than that used for the N-terminal marker. An amino acid sequence can also comprise an epitope which is recognized by coupling agents other than antibodies. One such sequence is 6 histidines sometimes referred to as a his-tag which binds to cobalt complex coupling agent.

A variety of N-terminal markers, affinity markers and C-terminal markers are available which can be used for this embodiment. The N-terminal marker could be BODIPY, affinity marker could be StrepTag and C-terminal marker could be a HisX6 tag. In this case, after translation, the reaction mixture is incubated in streptavidin

coated microtiter plate or with streptavidin coated beads. After washing unbound material, the N-terminal marker is directly measured using a fluorescence scanner while the C-terminal marker can be quantitated using anti-hisX6 antibodies conjugated with a fluorescent dye (like rhodamine or Texas Red) which has optical properties different than BODIPY, thus facilitating simultaneous dual detection.

In a different example, the N-terminal marker could be a biotin or photocleavable biotin incorporated by a misaminoacylated tRNA, the affinity marker could be a His X6 tag and the C-terminal had C-myc marker. In this case, after the translation, the reaction mixture is incubated with metal chelating beads or microtiter plates (for example Talon, ClonTech). After washing the unbound proteins, the plates or beads can be subjected to detection reaction using streptavidine conjugated fluorescence dye and C-myc antibody conjugated with other fluorescent dye. In addition, one can also use chemiluminescent detection method using antibodies which are conjugated with peroxidases.

It will be understood by those skilled in the area of molecular biology and biochemistry that the N-terminal marker, affinity marker and C-terminal marker can all consist of epitopes that can be incorporated into the nascent protein by designing the message or DNA coding for the nascent protein to have a nucleic acid sequence corresponding to the particular epitope. This can be accomplished using known methods such as the design of primers that incorporate the desired nucleic acid sequence into the DNA coding for the nascent protein using the polymerase chain reaction (PCR). It will be understood by those skilled in protein biochemistry that a wide variety of detection methods are available that can be used to detect both the N-terminal marker and the C-terminal markers. Additional examples include the use of chemiluminescence assays where an enzyme which converts a non-chemiluminescent substrate to a chemiluminescent product is conjugated to an antibody that is directed against a particular epitope.

One example of this approach is based on using a luminometer to measure luminous markers. A biotin detectable marker is incorporated at the N-terminal using a misaminoacylated tRNA. The biotin is detected by using a streptavidin which

is conjugated to *Renilla* luciferase from sea pansy. The C-terminal sequence comprises an epitope which interacts with a binding agent that has attached firefly luciferase. After separation of the nascent protein using a distinct epitope located near the N-terminal end of the protein, the protein is subjected to a dual luminescent luciferase assay based on a procedure described by Promega Corp and known as the Dual-Luciferase® Reporter Assay. This assay consists of first adding Luciferase Assay Reagent II available from Promega Corp. to the isolated nascent protein and then measuring the level of chemiluminescence. Stop & Glo® Reagent is then added which simultaneously quenches the firefly luminescence and activates the *Renilla* luminescence. The luciferase assay can be performed and quantified in seconds. A comparison of the level of luminescence measured from the firefly and *Renilla* luciferase provides an indication of whether a mutation is present or not in the coding message of the nascent protein.

In an additional example, the N-terminal marker comprises an affinity marker which is incorporated at the N-terminal end of the protein during its translation using a misaminoacylated tRNA. The affinity marker interacts with a coupling agent which acts to separate the nascent protein from the translation mixture. The nascent protein also contains a detectable marker which is located adjacent or close to the N-terminal of the protein containing the affinity marker. In addition, it contains a sequence specific marker at or near the C-terminal end of the protein. The detectable markers near the N-terminal and C-terminal ends of the nascent protein are then measured and compared to detect the presence of chain terminating or out-of-frame mutations.

There are a variety of additional affinity markers, N-terminal markers and C-terminal markers available for this embodiment. The affinity marker could be biotin or photocleavable biotin, N-terminal marker could be StepTag and C-terminal the C-myc epitope. In this case, after the translation, the reaction mixture is incubated with streptavidin coated beads or microtiter plates coated with streptavidin. After washing the unbound proteins, the plates or beads can be subjected to detection reaction using anti-his 6 antibodies conjugated with a fluorescent dye (like rhodamine or Texas Red) and C-myc antibody conjugated with other another fluorescent dye such as BODIPY.

In addition, one can also use chemiluminescent detection method using antibodies which are conjugated with peroxidases. Even in case of peroxidases conjugated antibodies, one can use fluorescent substrates and use FluorImager like device to quantitate N-terminal and C-terminal labels.

5 For optimal effectiveness, the N-terminal marker and affinity marker should be placed as close as possible to the N-terminal end of the protein. For example, if an N-terminal marker is incorporated using a misaminoacylated initiator, it will be located at the N-terminal amino acid. In this case, the affinity marker should be located immediately adjacent to the N-terminal marker. Thus, if a BODIPY marker which  
10 consists of a BODIPY conjugated to methionine is incorporated by a misaminoacylated initiator tRNA, it should be followed by an epitope sequence such as SteptTag (WSHPQFEK) (SEQ ID NO:8) so that the entire N-terminal sequence will be BODIPY-MWSPQFEK (SEQ ID NO: 10). However, for specific cases it may be advantageous to add intervening amino acids between the BODIPY-M and the epitope  
15 sequence in order to avoid interaction between the N-terminal marker and the affinity marker or the coupling agent which binds the affinity marker. Such interactions will vary depending on the nature of the N-terminal marker, affinity marker and coupling agent.

For optimal effectiveness, the C-terminal marker should be placed as close as  
20 possible to the C-terminal end of protein. For example, if a His-X6 tag is utilized, the protein sequence would terminate with 6 His. In some cases, an epitope may be located several residues before the C-terminal end of the protein in order to optimize the properties of the nascent protein. This might occur for example, if a specific amino acid sequence is necessary in order to modify specific properties of the nascent  
25 protein that are desirable such as its solubility or hydrophobicity.

In the normal application of this method, the ratio of the measured level of N-terminal and C-terminal markers for a nascent protein translated from a normal message can be used to calculate a standard normalized ratio. In the case of a message which may contain a mutation, deviations from this standard ratio can then be  
30 used to predict the extent of mutations. For example, where all messages are

defective, the ratio of the C-terminal marker to the N-terminal marker is expected to be zero. On the other hand, in the case where all messages are normal, the ratio is expected to be 1. In the case where only half of the message is defective, for example for a patient which is heterozygote for a particular genetic defect which is chain terminating or causes an out-of-frame reading error, the ratio would be 1/2.

There are several unique advantages of this method compared to existing techniques for detecting chain terminating or out-of-frame mutations. Normally, such mutations are detected by analyzing the entire sequence of the suspect gene using conventional DNA sequencing methods. However, such methods are time consuming, expensive and not suitable for rapid throughput assays of large number of samples. An alternative method is to utilize gel electrophoresis, which is able to detect changes from the expected size of a nascent protein. This approach, sometimes referred to as the protein truncation test, can be facilitated by using non-radioactive labeling methods such as the incorporation of detectable markers with misaminoacylated tRNAs. However, in many situations, such as high throughput screening, it would be desirable to avoid the use of gel electrophoresis which is time-consuming (typically 60-90 minutes). In the present method, the need for performing gel electrophoresis is eliminated. Furthermore, since the approach depends on comparison of two detectable signals from the isolated nascent protein which can be fluorescent, luminescent or some combination thereof, it is highly amenable to automation.

Measuring a sequence dependent marker located near the C-terminal end of the protein provides information about the presence of either a frameshift or chain terminating mutation since the presence of either would result in an incorrect sequence. The measurement of the N-terminal marker provides an internal control to which measurement of the C-terminal marker is normalized. Normalizing the level of the C-terminal marker to the N-terminal marker eliminates the inherent variabilities such as changes in the level of protein expression during translation that can undermine experimental accuracy. Separating the protein from the translation mixture using an affinity marker located at or close to the N-terminal end of the protein eliminates the occurrence of false starts which can occur when the protein is initiated.

during translation from an internal AUG in the coding region of the message. A false start can lead to erroneous results since it can occur after the chain terminating or out-of-frame mutation. This is especially true if the internal AUG is in-frame with the message. In this case, the peptide C-terminal marker will still be present even if message contains a mutation.

## B. Reporter Groups

Another embodiment of the invention is directed to a method for monitoring the synthesis of nascent proteins in a cellular or a cell-free protein synthesis system without separating the components of the system. These markers have the property that once incorporated into the nascent protein they are distinguishable from markers free in solution or linked to a tRNA. This type of marker, also called a reporter, provides a means to detect and quantitate the synthesis of nascent proteins directly in the cellular or cell-free translation system.

One type of reporters previously described in United States Patent 5,643,722 (hereby incorporated by reference) has the characteristic that once incorporated into the nascent protein by the protein synthesizing system, they undergo a change in at least one of their physical or physio-chemical properties. The resulting nascent protein can be uniquely detected inside the synthesis system in real time without the need to separate or partially purify the protein synthesis system into its component parts. This type of marker provides a convenient non-radioactive method to monitor the production of nascent proteins without the necessity of first separating them from pre-existing proteins in the protein synthesis system. A reporter marker would also provide a means to detect and distinguish between different nascent proteins produced at different times during protein synthesis by addition of markers whose properties are distinguishable from each other, at different times during protein expression. This would provide a means of studying differential gene expression.

One example of the utilization of reporters is schematically represented in Figure 10. A tRNA molecule is misaminoacylated with a reporter (R) which has lower or no fluorescence at a particular wavelength for monitoring and excitation. The

misaminoacylated tRNA is then introduced into a cellular or cell-free protein synthesis system and the nascent proteins containing the reporter analog are gradually produced. Upon incorporation of the reporter into the nascent protein (R\*), it exhibits an increased fluorescence at known wavelengths. The gradual production of the nascent protein is monitored by detecting the increase of fluorescence at that specific wavelength.

The chemical synthesis of a reporter can be based on the linkage of a chemical moiety or a molecular component having reporter properties with a native amino acid residue. There are many fluorescent molecules which are sensitive to their environment and undergo a change in the wavelength of emitted light and yield of fluorescence. When these chemical moieties, coupled to amino acids, are incorporated into the synthesized protein, their environments are altered because of a difference between the bulk aqueous medium and the interior of a protein which can cause reduced accessibility to water, exposure to charged ionic groups, reduced mobility, and altered dielectric constant of the surrounding medium. Two such examples are shown in Figure 11A.

One example of a reporter molecule is based on a fluorescent acridinium moiety and has the unique property of altering its emission properties, depending upon polarity or viscosity of the microenvironment. It has a higher quantum yield of fluorescence when subjected to hydrophobic environment and/or viscosity. Due to the hydrophobicity of the reporter itself, it is more likely to be associated with the hydrophobic core of the nascent protein after incorporation into the growing nascent polypeptide. An increase in the fluorescence intensity is a direct measure of protein synthesis activity of the translation system. Although, the environment of each reporter residue in the protein will be different, and in some cases, the reporter may be present on the surface of the protein and exposed to an aqueous medium, a net change occurs in the overall spectroscopic properties of the reporters incorporated into the protein relative to bulk aqueous medium. A change in the spectroscopic properties of only a subset of reporters in the protein will be sufficient to detect the synthesis of proteins that incorporate such reporters.







a second marker which is conjugated to the coupling agent.

One type of interaction between the markers that is advantageously used causes a fluorescence resonant energy transfer which occurs when the two markers are within a distance of between about 1 angstrom (Å) to about 50 Å, and preferably less than about 10 Å. In this case, excitation of one marker with electromagnetic radiation causes the second marker to emit electromagnetic radiation of a different wavelength which is detectable. This could be accomplished, for example, by incorporating a fluorescent marker at the N-terminal end of the protein using the *E. coli* initiator tRNA<sup>fmet</sup>. An epitope is then incorporated near the N-terminal end such as the SteptTag (WSHPQFEK) (SEQ ID NO:8) described by Sigma-Genosys. Streptavidin is then conjugated using known methods with a second fluorescent marker which is chosen to efficiently undergo fluorescent energy transfer with marker 1. The efficiency of this process can be determined by calculating the a Forster energy transfer radius which depends on the spectral properties of the two markers. The marker-streptavidin complex is then introduced into the translation mixture. Only when nascent protein is produced does fluorescent energy transfer between the first and second marker occur due to the specific interaction of the nascent protein SteptTag epitope with the streptavidin.

There are a variety of dyes which can be used as marker pairs in this method that will produce easily detectable signals when brought into close proximity. Previously, such dye pairs have been used for example to detect PCR products by hybridizing to probes labeled with a dye on one probe at the 5'-end and another at the 3'-end. The production of the PCR product brings a dye pair in close proximity causing a detectable FRET signal. In one application the dyes, fluorescein and LC 640 were utilized on two different primers (Roche Molecular Biochemicals- <http://www.biochem.boehringer-mannheim.com/lightcycler/monito03.htm>). When the fluorescein is excited by green light (around 500 nm) that is produced by a diode laser, the LC 640 emits red fluorescent light (around 640 nm) which can be easily detected with an appropriate filter and detector. In the case of nascent proteins, the pair of dyes BODIPY FL and LC 640 would function in a similar manner. For example,

incorporation of the BODIPY FL on the N-terminal end of the protein and the labeling of a binding agent with LC 640 which is directed against an N terminal epitope would allow detection of the production of nascent proteins.

5 The use of the marker pair BODIPY-FL and coumarin is a second pair which can be utilized advantageously. In one study, [Keller, R. C., Silvius, J. R., and De Kruijff, B. (1995) *Biochem Biophys Res Commun* 207(2), 508-14] it was found using the spectral overlap a Forster energy transfer radius (RO) of 50 +/- 2 A and 40 +/- 2 A for the coumarin- (beta-BODIPY FL ) and the coumarin-(beta-BODIPY 530/550 ) couple respectively. Experimentally this was estimated to be 49.0-51.5 A and 38.5-10 42.5 A respectively. It is also possible to use two markers with similar or identical spectral properties for the marker pair due to the process of quenching. For example, in one study this process was used in the case of BODIPY FL in order to study the processing of exogenous proteins in intact cells [Reis, R. C., Sorgine, M. H., and Coelho-Sampaio, T. (1998) *Eur J Cell Biol* 75(2), 192-7] and in a second case to15 study the kinetics of intracellular viral assembly [Da Poian, A. T., Gomes, A. M., and Coelho-Sampaio, T. (1998) *J Virol Methods* 70(1), 45-58].

As stated above, a principal advantage of using reporters is the ability to monitor the synthesis of proteins in cellular or a cell-free translation systems directly without further purification or isolation steps. Reporter markers may also be utilized in20 conjunction with cleavable markers that can remove the reporter property at will. Such techniques are not available using radioactive amino acids which require an isolation step to distinguish the incorporated marker from the unincorporated marker. With *in vitro* translation systems, this provides a means to determine the rate of synthesis of proteins and to optimize synthesis by altering the conditions of the25 reaction. For example, an *in vitro* translation system could be optimized for protein production by monitoring the rate of production of a specific calibration protein. It also provides a dependable and accurate method for studying gene regulation in a cellular or cell-free systems.

### C. Affinity Markers

Another embodiment of the invention is directed to the use of markers that facilitate the detection or separation of nascent proteins produced in a cellular or cell-free protein synthesis system. Such markers are termed affinity markers and have the property that they selectively interact with molecules and/or materials containing acceptor groups. The affinity markers are linked by aminoacylation to tRNA molecules in an identical manner as other markers of non-native amino acid analogs and derivatives and reporter-type markers as described. These affinity markers are incorporated into nascent proteins once the misaminoacylated tRNAs are introduced into a translation system.

An affinity marker facilitates the separation of nascent proteins because of its selective interaction with other molecules which may be biological or non-biological in origin through a coupling agent. For example, the specific molecule to which the affinity marker interacts, referred to as the acceptor molecule, could be a small organic molecule or chemical group such as a sulfhydryl group (-SH) or a large biomolecule such as an antibody. The binding is normally chemical in nature and may involve the formation of covalent or non-covalent bonds or interactions such as ionic or hydrogen bonding. The binding molecule or moiety might be free in solution or itself bound to a surface, a polymer matrix, or a residue on the surface of a substrate. The interaction may also be triggered by an external agent such as light, temperature, pressure or the addition of a chemical or biological molecule which acts as a catalyst.

The detection and/or separation of the nascent protein and other preexisting proteins in the reaction mixture occurs because of the interaction, normally a type of binding, between the affinity marker and the acceptor molecule. Although, in some cases some incorporated affinity marker will be buried inside the interior of the nascent protein, the interaction between the affinity marker and the acceptor molecule will still occur as long as some affinity markers are exposed on the surface of the nascent protein. This is not normally a problem because the affinity marker is distributed over several locations in the protein sequence.

Affinity markers include native amino acids, non-native amino acids, amino

acid derivatives or amino acid analogs in which a coupling agent is attached or incorporated. Attachment of the coupling agent to, for example, a non-native amino acid may occur through covalent interactions, although non-covalent interactions such as hydrophilic or hydrophobic interactions, hydrogen bonds, electrostatic interactions or a combination of these forces are also possible. Examples of useful coupling agents include molecules such as haptens, immunogenic molecules, biotin and biotin derivatives, and fragments and combinations of these molecules. Coupling agents enable the selective binding or attachment of newly formed nascent proteins to facilitate their detection or isolation. Coupling agents may contain antigenic sites for a specific antibody, or comprise molecules such as biotin which is known to have strong binding to acceptor groups such as streptavidin. For example, biotin may be covalently linked to an amino acid which is incorporated into a protein chain. The presence of the biotin will selectively bind only nascent proteins which incorporated such markers to avidin molecules coated onto a surface. Suitable surfaces include resins for chromatographic separation, plastics such as tissue culture surfaces for binding plates, microtiter dishes and beads, ceramics and glasses, particles including magnetic particles, polymers and other matrices. The treated surface is washed with, for example, phosphate buffered saline (PBS), to remove non-nascent proteins and other translation reagents and the nascent proteins isolated. In some case these materials may be part of biomolecular sensing devices such as optical fibers, chemfets, and plasmon detectors.

One example of an affinity marker is dansyllysine (Figure 5). Antibodies which interact with the dansyl ring are commercially available (Sigma Chemical; St. Louis, MO) or can be prepared using known protocols such as described in *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, editors, Cold Spring Harbor Laboratory Press, 1988) which is hereby specifically incorporated by reference. Many conventional techniques exist which would enable proteins containing the dansyl moiety to be separated from other proteins on the basis of a specific antibody-dansyl interaction. For example, the antibody could be immobilized onto the packing material of a chromatographic column. This method, known as affinity column

chromatography, accomplishes protein separation by causing the target protein to be retained on the column due to its interaction with the immobilized antibody, while other proteins pass through the column. The target protein is then released by disrupting the antibody-antigen interaction. Specific chromatographic column materials such as ion-exchange or affinity Sepharose, Sephacryl, Sephadex and other chromatography resins are commercially available (Sigma Chemical; St. Louis, MO; Pharmacia Biotech; Piscataway, NJ).

Separation can also be performed through an antibody-dansyl interaction using other biochemical separation methods such as immunoprecipitation and immobilization of the antibodies on filters or other surfaces such as beads, plates or resins. For example, protein could be isolated by coating magnetic beads with a protein-specific antibody. Beads are separated from the extract using magnetic fields. A specific advantage of using dansyllysine as an affinity marker is that once a protein is separated it can also be conveniently detected because of its fluorescent properties.

In addition to antibodies, other biological molecules exist which exhibit equally strong interaction with target molecules or chemical moieties. An example is the interaction of biotin and avidin. In this case, an affinity analog which contains the biotin moiety would be incorporated into the protein using the methods which are part of the present invention. Biotin-lysine amino acid analogs are commercially available (Molecular Probes; Eugene, OR).

Affinity markers also comprise one component of a multicomponent complex which must be formed prior to detection of the marker. One particular embodiment of this detection means involves the use of luminescent metal chelates, in particular luminescent rare earth metal chelates. It is well known that certain molecules form very stable complexes with rare earth metals. It is also well known that introduction of chromophore into these chelates sensitizes luminescence of these complexes. A variety of detection schemes based on the use of luminescent rare earth metal chelates have been described: Hemmila, I.A., "Applications of Fluorescence in Immunoassays", (Wiley&Sons 1991).

In a preferred embodiment, tRNA is misaminoacylated with a chromophore

that also acts as a rare earth shelter. This modified aminoacyl tRNA is then introduced into cellular or cell-free protein translation system and the modified amino acid incorporated into nascent protein. The mixture is then separated using gel electrophoresis and the gel is incubated with a solution containing rare earth cation. Under these conditions rare earth cations form luminescent complexes with amino acids modified with a chelator present only in nascent proteins. The nascent proteins are then detected using a mid-range UV transilluminator (350 nm), which excites the formed lanthanide complex. The image is then recorded using polaroid camera or CCD array and a filter. In one embodiment, the derivatives of salicylic acid as one component and terbium ions as a second component of the binary detection system are used.

Affinity markers can also comprise cleavable markers incorporating a coupling agent. This property is important in cases where removal of the coupled agent is required to preserve the native structure and function of the protein and to release nascent protein from acceptor groups. In some cases, cleavage and removal of the coupling agent results in production of a native amino acid. One such example is photocleavable biotin coupled to an amino acid.

Photocleavable biotin contains a photoreactive moiety which comprises a phenyl ring derivatized with functionalities represented in Figure 12 by X, Y and Z. X allows linkage of PCB to the bimolecular substrate through the reactive group X'. Examples of X' include Cl, O-N-hydroxysuccinimidyl, OCH<sub>2</sub>CN, OPhF<sub>3</sub>, OPhCl<sub>3</sub>, N<sub>3</sub>. Y represents a substitution pattern of a phenyl ring containing one or more substitutions such as nitro or alkoxyl. The functionality Z represents a group that allows linkage of the cross-linker moiety to the photoreactive moiety. The photoreactive moiety has the property that upon illumination, it undergoes a photoreaction that results in cleavage of the PCB molecule from the substrate.

A lysine-tRNA is misaminoacylated with photocleavable biotin-lysine, or chemically modified to attach a photocleavable biotin amino acid. The misaminoacylated tRNA is introduced into a cell-free protein synthesizing system and nascent proteins produced. The nascent proteins can be separated from other



components of the system by streptavidin-coated magnetic beads using conventional methods which rely on the interaction of beads with a magnetic field. Nascent proteins are released then from beads by irradiation with UV light of approximately 280 nm wavelength.

5 Many devices designed to detect proteins are based on the interaction of a target protein with specific immobilized acceptor molecule. Such devices can also be used to detect nascent proteins once they contain affinity markers such as biodetectors based on sensing changes in surface plasmons, light scattering and electronic properties of materials that are altered due to the interaction of the target molecule with the  
10 immobilized acceptor group.

Nascent proteins, including those which do not contain affinity-type markers, may be isolated by more conventional isolation techniques. Some of the more useful isolation techniques which can be applied or combined to isolate and purify nascent proteins include chemical extraction, such as phenol or chloroform extract, dialysis,  
15 precipitation such as ammonium sulfate cuts, electrophoresis, and chromatographic techniques. Chemical isolation techniques generally do not provide specific isolation of individual proteins, but are useful for removal of bulk quantities of non-proteinaceous material. Electrophoretic separation involves placing the translation mixture containing nascent proteins into wells of a gel which may be a denaturing or  
20 non-denaturing polyacrylamide or agarose gel. Direct or pulsed current is applied to the gel and the various components of the system separate according to molecular size, configuration, charge or a combination of their physical properties. Once distinguished on the gel, the portion containing the isolated proteins removed and the nascent proteins purified from the gel. Methods for the purification of protein from  
25 acrylamide and agarose gels are known and commercially available.

Chromatographic techniques which are useful for the isolation and purification of proteins include gel filtration, fast-pressure or high-pressure liquid chromatography, reverse-phase chromatography, affinity chromatography and ion exchange  
30 chromatography. These techniques are very useful for isolation and purification of proteins species containing selected markers.

Another embodiment of the invention is directed to the incorporation of non-native amino acids or amino acid derivatives with marker or affinity properties at the amino-terminal residue of a nascent protein (Figure 13). This can be accomplished by using the side chain of an amino acid or by derivatizing the terminal amino group of an amino acid. In either case the resulting molecule is termed an amino acid derivative. The amino-terminal residue of a protein is free and its derivatization would not interfere with formation of the nascent polypeptide. The non-native amino acid or amino acid derivative is then used to misaminoacylate an initiator tRNA which only recognizes the first AUG codon signaling the initiation of protein synthesis. After introduction of this misaminoacylated initiator tRNA into a protein synthesis system, marker is incorporated only at the amino terminal of the nascent protein. The ability to incorporate at the N-terminal residue can be important as these nascent molecules are most likely to fold into native conformation. This can be useful in studies where detection or isolation of functional nascent proteins is desired.

Not all markers incorporated with misaminoacylated initiator tRNAs at the amino-terminal residue of the nascent protein show the same acceptance by the protein translational machinery. Furthermore, the range of incorporation of different markers can be more restrictive compared to the use of non-initiator tRNAs such as lysyl-tRNA. Although the factors influencing this discrimination between markers for incorporation by a misaminoacylated initiator tRNA are not fully elucidated, one possibility is that the initiation factor (IF2) which is used for carrying formylmethionine-tRNA<sup>fmet</sup> to ribosomes plays a role. A second possibility is that the interaction between the marker structure and the ribosomes plays a role. For example, the marker, BODIPY-FL is accepted by the protein translational machinery to a greater extent than smaller fluorescent markers such as NBD. For this reason, BODIPY-FL is a superior marker for use in the detection of nascent protein when incorporated through initiator tRNAs.

A marker group can also be incorporated at the N terminal by using a mutant tRNA which does not recognize the normal AUG start codon. In some cases this can lead to a higher extent of specific incorporation of the marker. For example, the

mutant of initiator tRNA, where the anticodon has been changed from CAU--> CUA (resulting in the change of initiator methionine codon to amber stop codon) has shown to act as initiator suppressor tRNA (Varshney U, RajBhandary UL, Proc Natl Acad Sci U S A 1990 Feb;87(4):1586-90; Initiation of protein synthesis from a termination codon). This tRNA initiates the protein synthesis of a particular gene when the normal initiation codon, AUG is replaced by the amber codon UAG. Furthermore, initiation of protein synthesis with UAG and tRNA(fMet<sup>CUA</sup>) was found to occur with glutamine and not methionine. In order to use this tRNA to introduce a marker at the N terminal of a nascent protein, this mutant tRNA can be enzymatically aminoacylated with glutamine and then modified with suitable marker. Alternatively, this tRNA could be chemically aminoacylated using modified amino acid (for example methionine-BODIPY). Since protein translation can only be initiated by this protein on messages containing UAG, all proteins will contain the marker at the N-terminal end of the protein.

#### **D. Mass Spectrometry**

Mass spectrometry measures the mass of a molecule. The use of mass spectrometry in biology is continuing to advance rapidly, finding applications in diverse areas including the analysis of carbohydrates, proteins, nucleic acids and biomolecular complexes. For example, the development of matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS) has provided an important tool for the analysis of biomolecules, including proteins, oligonucleotides, and oligosacharrides [Karas, 1987 #6180; Hillenkamp, 1993 #6175]. This technique's success derives from its ability to determine the molecular weight of large biomolecules and non-covalent complexes (>500,000 Da) with high accuracy (0.01%) and sensitivity (subfemtomole quantities). Thus far, it has been found applicable in diverse areas of biology and medicine including the rapid sequencing of DNA, screening for bioactive peptides and analysis of membrane proteins.

Markers incorporated by misaminoacylated tRNAs into nascent proteins, especially at a specific position such at the N-terminal can be used for the detection

of nascent proteins by mass spectrometry. Without such a marker, it can be very difficult to detect a band due to a nascent protein synthesized in the presence of a cellular or cell-free extract due the presence of many other molecules of similar mass in the extract. For example, in some cases less than 0.01% of the total protein mass of the extract may comprise the nascent protein(s). Furthermore, molecules with similar molecular weight as the nascent protein may be present in the mixture. Such molecules will overlap with peaks due to the nascent protein. This problem is particularly severe if the nascent protein is a transcription or translation factor already present in the cell-free or cellular protein synthesis. The synthesis of additional amounts of this protein in the protein synthesis system would be difficult to detect using known methods in mass spectrometry since peak intensities are not correlated in a linear manner with protein concentration.

Detection by mass spectrometry of a nascent protein produced in a translation system is also very difficult if the mass of the nascent protein produced is not known. This might situation might occur for example if the nascent protein is translated from DNA where the exact sequence is not known. One such example is the translation of DNA from individuals which may have specific mutations in particular genes or gene fragments. In this case, the mutation can cause a change in the protein sequence and even result in chain truncation if the mutation results in a stop codon. The mass of nascent proteins produced in a translation system might also not be known if DNA is derived from unknown sources such as as colonies of bacteria which can contain different members of a gene library or fragments thereof.

In one embodiment of the invention, the incorporation of markers of a specific mass (mass markers) into nascent proteins can be used to eliminate all of the above mentioned problems associated with the conventional mass spectrometric approach. First, a tRNA misaminoacylated with a marker of a known mass is added to the protein synthesis system. The synthesis system is then incubated to produce the nascent proteins. The mass spectrum of the protein synthesis system is then measured. The presence of the nascent protein can be directly detected by identifying peaks in the mass spectrum of the protein synthesis system which correspond to the

mass of the unmodified protein and a second band at a higher mass which corresponds to the mass of the nascent protein plus the modified amino acid containing the mass of the marker.

5 There are several steps that can be taken to optimize the efficient detection of nascent proteins using this method. The mass of the marker should exceed the resolution of the mass spectrometer, so that the increased in mass of the nascent protein can be resolved from the unmodified mass. For example, a marker with a mass exceeding 100 daltons can be readily detected in proteins with total mass up to 100,000 using both matrix assisted laser desorption (MALDI) or electrospray  
10 ionization (ESI) techniques. The amount of misaminoacylated tRNA should be adjusted so that the incorporation of the mass marker occurs in approximately 50% of the total nascent protein produced. An initiator tRNA is preferable for incorporation of the mass marker since it will only be incorporated at the N-terminal of the nascent protein, thus avoiding the possibility that the nascent protein will contain multiple  
15 copies of the mass marker.

One example of this method is the incorporation of the marker BODIPY-FL, which has a mass of 282, into a nascent protein using a misaminoacylated initiator tRNA. Incorporation of this marker into a nascent protein using a misaminoacylated initiator tRNA causes a band to appear at approximately 282 daltons above the normal  
20 band which appears for the nascent protein. Since the incorporation of the marker is less than one per protein due to competition of non-misaminoacylated E.coli tRNA<sup>fmet</sup>, a peak corresponding to the unmodified protein also appears. Identification of these two bands separated by the mass of the marker allows initial identification of the band due to the nascent protein. Further verification of the band due to the nascent protein  
25 can be made by adjusting the level of the misaminoacylated initiator tRNA in the translation mixture. For example, if the misaminoacylated initiator tRNA is left out, than only a peak corresponding to the unmodified protein appears in the mass spectrum of the protein synthesis system. By comparing the mass spectrum from the  
30 protein synthesis system containing and not containing the misaminacylated tRNA with the BODIPY-FL, the presence of the nascent protein can be uniquely identified, even



in a cell or cell-free translation system.

Markers with affinity properties which are incorporated by misaminoacylated tRNAs into nascent proteins can also be very useful for the detection of such proteins by mass spectrometry. Such markers can be used to isolate nascent proteins from the rest of the cell-free or cellular translation system. In this case, the isolation of the nascent proteins from the rest of the cell-free mixture removes interference from bands due to other molecules in the protein translation system. An example of this approach is the incorporation of photocleavable biotin into the N-terminal end of a nascent proteins using misaminoacylated tRNA. When this marker is incorporated onto the N-terminal end of a nascent protein using an *E. coli* tRNA<sup>met</sup>, it provides a convenient affinity label which can be bound using streptavidin affinity media such as streptavidin agarose. Once the nascent protein is separated by this method from the rest of the protein synthesis system, it can be released by UV-light and analyzed by mass spectrometry. In the case of MALDI mass spectrometry, release of the nascent protein can most conveniently be accomplished by using the UV-laser excitation pulses of the MALDI system. Alternatively, the sample can be irradiated prior to mass spectrometric analysis in the case of MALDI or ESI mass spectrometry.

#### **E. Electrophoresis**

Another embodiment of the invention is directed to methods for detecting by electrophoresis the interaction of molecules or agents with nascent proteins which are translated in a translation system. This method allows a large number of compounds or agents to be rapidly screened for possible interaction with the expressed protein of specific genes, even when the protein has not been isolated or its function identified. It also allows a library of proteins expressed by a pool of genes to be rapidly screened for interaction with compounds or agents without the necessity of isolating these proteins or agents. The agents might be part of a combinatorial library of compounds or present in a complex biological mixture such as a natural sample. The agents might interact with the nascent proteins by binding to them or to cause a change in the structure of the nascent protein by chemical or enzymatic modification.

In addition to gel electrophoresis, which measures the electrophoretic mobility of proteins in gels such as polyacrylamide gel, this method can be performed using capillary electrophoresis. CE measures the electrophoretic migration time of a protein which is proportional to the charge-to-mass ratio of the molecule. One form of CE, sometimes termed affinity capillary electrophoresis, has been found to be highly sensitive to interaction of proteins with other molecules including small ligands as long as the binding produces a change in the charge-to-mass ratio of the protein after the binding event. The highest sensitivity can be obtained if the protein is conjugated to a marker with a specifically detectable electromagnetic spectral property such as a fluorescent dye. Detection of a peak in the electrophoresis chromatogram is accomplished by laser induced emission of mainly visible wavelengths. Examples of fluorescent dyes include fluorescein, rhodamine, Texas Red and BODIPY.

It is very difficult to detect a nascent protein synthesized in a cellular or cell-free extract by CE without subsequent isolation and labeling steps due the need for high sensitivity detection and the presence of many other molecules of similar mass/charge ratio in the extract. For example, in typical cases less than 0.01% of the total protein mass of the extract may comprise the nascent protein(s). Other molecules with similar electrophoretic migration times as the nascent protein may be present in the mixture. Such molecules will overlap with peaks due to the nascent protein.

It is also very difficult using conventional methods of CE to detect the interaction of molecules with nascent proteins produced in a cell free or cellular synthesis system. Affinity capillary electrophoresis has been found to be sensitive to interaction of proteins with other molecules including small ligands as long as the binding produces a change in the charge-to-mass ratio of the protein after the binding event. However, the selective addition of a marker such as a fluorescent dye to a nascent protein is not possible using conventional means because most markers reagents will nonspecifically label other molecules in the protein synthesis system besides the nascent proteins. In some cases, it may be possible utilize a specific substrate or ligand which binds only to the nascent protein. However this approach requires a detailed knowledge of the binding properties of the nascent protein and



special design of a ligand with marker properties. The nascent protein may also be isolated from the protein synthesis system and then selectively labeled with a detectable marker. However, this also requires the development of a procedure for isolation of the nascent protein which can be time consuming and requires extensive knowledge of properties of the protein or protein engineering to incorporate an affinity epitope. Even after a nascent protein has been isolated, it is often difficult to uniformly label the protein with a marker so that the charge/mass ratio of each labeled protein remains the same. In the most advantageous form of labeling, a highly fluorescent marker is incorporated at only one specific position in the protein thus avoiding a set of proteins with different electrophoretic mobilities.

The method of the invention also overcomes major problems associated with the rapid screening of samples for new therapeutic compounds using capillary electrophoresis (CE) such as described in US. Patent 5,783,397 (hereby incorporated by reference) when the target protein is a nascent protein expressed in a translation system. This includes the need to uniformly label expressed target proteins in a translation system with markers for high sensitivity analysis by CE which normally requires lengthy isolation steps and special techniques for uniform labeling.

The method can also be used in conjunction with expression cloning method for isolating novel cDNA clones such as described in U.S. patent 565,451, which is specifically incorporated by reference. This patent describes novel methods to identify cDNA clones by a) collecting pools of about 100 individual bacterial colonies; and b) expressing proteins encoded by the cDNAs in the pools *in vitro*. Proteins which can be identified in this manner include but are not limited to nucleic acid binding protein, cytoskeletal protein, growth factor, differentiation factor, post-translationally modified protein, phosphorylated protein, proteolytically cleaved protein, glycosylated protein, subunit or a multiple component of a protein complex, enzyme, isoform of a known protein, mutant form of known protein. Importantly, the method includes as a crucial step identifying a desired protein from protein translation system. Two such methods described for identifying the protein involve radioactive labeling and chemical labeling. However, these steps can be extremely time-consuming and are not

conducive to rapidly screening an extract for the desired protein.

The present invention avoids all of the problems discussed above. In one embodiment of the invention a tRNA misaminoacylated with a detectable marker is added to the protein synthesis system. The system is incubated to incorporate the detectable marker into the nascent proteins. One or more molecules (agents) are then combined with the nascent proteins (either before or after isolation) to allow agents to interact with nascent proteins. Aliquots of the mixture are then subjected to electrophoresis. Nascent proteins which have interacted with the agents are identified by detecting changes in the electrophoretic mobility of nascent proteins with incorporated markers. In the case where the agents have interacted with the nascent proteins, the proteins can be isolated and subsequently subjected to further analysis. In cases where the agents have bound to the nascent proteins, the bound agents can be identified by isolating the nascent proteins.

In one example of this method, the fluorescent marker BODIPY-FL is used to misaminoacylate an *E. coli* initiator tRNA<sup>finet</sup> as previously described. The misaminoacylated tRNA is then added to a protein synthesis system and the system incubated to produce nascent protein containing the BODIPY-FL at the N-terminal. A specific compound which may bind to the nascent protein is then added to the protein synthesis system at a specific concentration. An aliquot from the mixture is then injected into an apparatus for capillary electrophoresis. Nascent proteins in the mixture are identified by detection of the fluorescence from the BODIPY-FL using exciting light from an Argon laser tuned to 488 nm. Interaction of the specific compound is determined by comparing the electrophoretic mobility measured of the nascent protein exposed to the specific compound with a similar measurement of the nascent protein that has not been exposed. The binding strength of the compound can then be ascertained by altering the concentration of the specific compounds added to the protein synthesis system and measuring the change in the relative intensity of bands assigned to the uncomplexed and complexed nascent protein.

The method is not limited to studying the interaction of one agent with one nascent protein translated in a protein translation system. For example, a library of

compounds can be screened to identify those which serve as ligands for specific target protein. In addition to interactions which involve the binding of one or more agents to the nascent proteins interactions which result in a modification of the nascent protein including but not limited to phosphorylation, proteolysis, glycosylation, formation of a complex with other biological molecules can be detected using the marker incorporated in the nascent proteins when combined with electrophoresis. For example, the interaction of an antibody with the nascent proteins can be detected due to a change in the effective electrophoretic mobility of the complex formed. A similar approach could be used to identify the presence of one or more compounds in a complex mixture which bind to the nascent protein. Such a mixture might constitute a library of compounds produced by combinatorial chemistry or compounds which might be present in a complex biological mixture such as natural samples which may contain therapeutic compounds.

#### **F. Microscale Methods**

While the present invention contemplates capillary electrophoresis (see above), other methods are also contemplated. In particular, microscale methods can be employed in conjunction with the novel markers (e.g. BODIPY) and methods of the present invention. The methods are "microscale" in that the dimensions of the channels on the device (and the corresponding fluid volumes) are very small (typically in the picometer range). For example, channels are typically between approximately 0.10 and 0.50  $\mu\text{m}$  in depth and between approximately 5 and 500  $\mu\text{m}$  in width.

Although there are many formats, materials, and size scales for constructing integrated fluidic systems, the present invention contemplates microfabricated devices as a solution to the many inefficiencies of larger scale screening. Devices can be microfabricated from a number of materials. Silicon is the material used for the construction of computing microprocessors and its fabrication technologies have developed at an unprecedented pace over the past 30 years. The principal modern method for fabricating semiconductor integrated circuits is the so-called planar process. The planar process relies on the unique characteristics of silicon and comprises a

complex sequence of manufacturing steps involving deposition, oxidation, photolithography, diffusion and/or ion implantation, and metallization, to fabricate a "layered" integrated circuit device in a silicon substrate. *See e.g.*, W. Miller, U.S. Patent No. 5,091,328, hereby incorporated by reference. While this technology was initially applied to making microelectronic devices, the same techniques are currently being used for micromechanical systems.

Continuous flow liquid transport has been described using a microfluidic device developed with silicon. *See J. Pfahler et al.*, *Sensors and Actuators*, A21-A23 (1990), pp. 431-434. Pumps have also been described, using external forces to create flow, based on micromachining of silicon. *See H.T.G. Van Lintel et al.*, *Sensors and Actuators* 15:153-167 (1988). SDS capillary gel electrophoresis of proteins in microfabricated channels has also been described. *See Yao S et al.*, "SDS capillary gel electrophoresis of proteins in microfabricated channels," *PNAS* 96:5372 (1999). Compared to more conventional two-dimensional denaturing gel electrophoresis (which is generally time consuming and requires considerable amounts of sample), this microchannel-based separation technique was shown to be quick and offer high resolution.

As a mechanical building material, silicon has well-known fabrication characteristics. The economic attraction of silicon devices is that their associated micromachining technologies are, essentially, photographic reproduction techniques. In these processes, transparent templates or masks containing opaque designs are used to photodefine objects on the surface of the silicon substrate. The patterns on the templates are generated with computer-aided design programs and can delineate structures with line-widths of less than one micron. Once a template is generated, it can be used almost indefinitely to produce identical replicate structures. Consequently, even extremely complex micromachines can be reproduced in mass quantities and at low incremental unit cost — provided that all of the components are compatible with the silicon micromachining process. While the present invention contemplates other substrates, such as glass or quartz, for use in photolithographic methods to construct microfabricated analysis devices, silicon is preferred because of the added advantage of

allowing a large variety of electronic components to be fabricated within the same structure.

In one embodiment, the present invention contemplates silicon micromachined components in an integrated analysis system. Sample (e.g. a test compound) and one  
5 or more reagents (e.g. a BODIPY labelled nascent protein) are injected either continuously or in pulses into the device through entry ports and they are transported through channels to a reaction chamber, such as a thermally controlled reactor where mixing and reactions take place. The biochemical products can be then moved down a new channel (or by an electrophoresis module, if desired) where migration data is  
10 collected by a detector and transmitted to a recording instrument. If desired, a polymer can be used in the channels to provide resolution by molecular sieving. The biochemical products can be isolated by diverting the flow to an external port for subsequent additional analysis. Importantly, the fluidic and electronic components are designed to be fully compatible in function and construction with the biological  
15 reactions and reagents. In this embodiment, potential test compounds can be rapidly screened for interaction with a labeled nascent protein or multiple nascent proteins that are co-expressed in a translation reaction system. In this manner the system can be used to screen for interaction so as to identify useful drugs.

In another embodiment, one or more components of the protein synthesis  
20 system are introduced into the device through entry ports and they are transported through channels to a reaction chamber, such as a thermally controlled reactor, where the expression of the nascent protein which contains the marker such as BODIPY occurs. The labeled nascent protein can then be mixed with one or more reagents (e.g. a test compound) that are introduced into the device through entry ports. After the  
25 reaction takes place, the biochemical products can be then moved down a new channel (or by an electrophoresis module, if desired) where migration data is collected by a detector and transmitted to a recording instrument. It is to be understood that components of the protein synthesis system which can be introduced into the device can include misaminoacylated tRNAs, DNA, mRNA, amino acids and nucleotides.  
30 The components can be introduced either continuously or in discrete pulses. The DNA

may also be produced within the micromachined device by enzymatic reactions such as the polymerase chain reaction as has been described. See Kopp *et al.*, "Chemical Amplification: Continuous Flow PCR on a Chip," *Science* 280:1046 (1998).

In silicon micromachining, a simple technique to form closed channels involves etching an open trough on the surface of a substrate and then bonding a second, unetched substrate over the open channel. There are a wide variety of isotropic and anisotropic etch reagents, either liquid or gaseous, that can produce channels with well-defined side walls and uniform etch depths. Since the paths of the channels are defined by the photo-process mask, the complexity of channel patterns on the device is virtually unlimited. Controlled etching can also produce sample entry holes that pass completely through the substrate, resulting in entry ports on the outside surface of the device connected to channel structures.

#### G. Multiple Misaminoacylated tRNAs

It may often be advantageous to incorporate more than one marker into a single species of protein. This can be accomplished by using a single tRNA species such as a lysine tRNA misaminoacylated with both a marker such as dansyllysine and a coupling agent such as biotin-lysine. Alternatively, different tRNAs which are each misaminoacylated with different markers can also be utilized. For example, the coumarin derivative could be used to misaminoacylate a tryptophan tRNA and a dansyl-lysine used to misaminoacylate a lysine tRNA.

One use of multiple misaminoacylated tRNAs is to study the expression of proteins under the control of different genetic elements such as repressors or activators, or promoters or operators. For example, the synthesis of proteins at two different times in response to an internal or external agent could be distinguished by introducing misaminoacylated tRNAs at different times into the cellular or cell-free protein synthesis system. A tRNA<sup>tr</sup> might be charged with marker A and a tRNA<sup>lys</sup> charged with marker B, yielding A-tRNA<sup>tr</sup> and B-tRNA<sup>lys</sup>, respectively. In this case, protein one under the control of one promoter can be labeled by adding the A-tRNA<sup>tr</sup> to the reaction system. If a second misaminoacylated tRNA, B-tRNA<sup>lys</sup> is then added and a

second promoter for protein two activated, the nascent protein produced will contain both label A and B. Additional markers could also be added using additional tRNA molecules to further study the expression of additional proteins. The detection and analysis of multiply labeled nascent proteins can be facilitated by using the multi-colored electrophoresis pattern reading system, described in U.S. Patent No. 5,190,632, which is specifically incorporated by reference, or other multi-label reading systems such as those described in U.S. Patent Nos. 5,069,769 and 5,137,609, which are both hereby specifically incorporated by reference.

A second use of multiple misaminoacylated tRNAs is in the combined isolation and detection of nascent proteins. For example, biotin-lysine marker could be used to misaminoacylate one tRNA and a coumarin marker used to misaminoacylate a different tRNA. Magnetic particles coated with streptavidin which binds the incorporated lysine-biotin would be used to isolate nascent proteins from the reaction mixture and the coumarin marker used for detection and quantitation.

A schematic diagram of the basics of the above methods is shown in Figure 14. In a first step, the marker selected (M), which may have reporter (R) or affinity (A) properties, is chemically or enzymatically misaminoacylated to a single tRNA species or a mixture of different tRNAs. Prior to protein synthesis, a predetermined amount of the misaminoacylated tRNA, charged with the fluorescent marker is mixed with the cell-free protein synthesis reaction system at concentrations sufficient to allow the misaminoacylated tRNA to compete effectively with the corresponding tRNA. After an incubation of about 1-3 hours, the reaction mixture is analyzed using conventional polyacrylamide or agarose gel electrophoresis. After electrophoresis, the gel is illuminated by UV radiation. Bands due to the nascent protein exhibit distinct fluorescence and can be easily and rapidly distinguished, either visually or photographically, from non-fluorescent bands of preexisting proteins. Nascent proteins can be isolated by excising the fluorescent band and electroeluting the protein from the extracted gel pieces. The quantities and molecular weights of the nascent proteins can be determined by comparison of its fluorescence with the fluorescence produced by a set of proteins with known molecular weights and known quantities. The results of the

assay can be recorded and stored for further analysis using standard electronic imaging and photographic or spectroscopic methods.

#### **H. Resulting Compositions**

Another embodiment of the invention is directed to a composition comprising nascent proteins isolated or purified by conventional methods after translation in the presence of markers. Compositions can be utilized in manufacturing for the preparation of reagents such as coatings for tissue culture products and in the pharmaceutical industry.

Incorporation of markers into nascent proteins utilized in manufacturing facilitates analysis of the final manufactured product or process by detection of marker. For example, nascent proteins produced may be used as coatings for tissue culture products. The reproducibility of a particular coating process could be accurately determined by detecting variations of marker emissions over the surface of the coated product. In addition, non-toxic markers incorporated into proteins encompassed within a pharmaceutical preparation such as a hormone, steroid, immune product or cytokine can be utilized to facilitate safe and economical isolation of that protein preparation. Such products could be used directly without the need for removal of marker. When very low concentrations of marker are preferred, limiting amounts of marked proteins could be used to follow a protein through a purification procedure. Such proteins can be efficiently purified and the purity of the resulting composition accurately determined. In addition, the presence of markers may facilitate study and analysis of pharmaceutical compositions in testing. For example, markers can be utilized to determine serum half-life, optimum serum levels and the presence or absence of breakdown products of the composition in a patient.

Alternatively, nascent proteins may contain specific markers which serve as therapeutically useful compounds such as toxic substances. These proteins are administered to a patient and the therapeutic moiety released after proteins have identified and possibly bound to their respective targets. Release may be electrical stimulation, photochemical cleavage or other means whereby the moiety is specifically





bacteria in biological samples containing prokaryotic cells. Kits would contain translation reagents necessary to synthesize protein plus tRNA molecules charged with detectable non-radioactive markers. The addition of a biological sample containing the bacteria-specific genes would supply the nucleic acid needed for translation. Bacteria from these samples would be selectively lysed using a bacteria directed toxin such as Colicin E1 or some other bacteria-specific permeabilizing agent. Specific genes from bacterial DNA could also be amplified using specific oligonucleotide primers in conjunction with polymerase chain reaction (PCR), as described in U.S. Patent No. 4,683,195, which is hereby specifically incorporated by reference. Nascent proteins containing marker would necessarily have been produced from bacteria. Utilizing additional markers or additional types of detection kits, the specific bacterial infection may be identified.

The present invention also contemplates kits which permit the GFTT described above. For example, the present invention contemplates kits to detect specific diseases such as familial adenomatous polyposis. In about 30 to 60% of cases of familial adenomatous polyposis, the diseased tissues also contain chain terminated or truncated transcripts of the APC gene (S.M. Powell et al., N. Engl. J. Med. 329:1982-87, 1993). Chain termination occurs when frameshift cause a stop codon such as UAG, UAA or UGA to appear in the reading frame which terminates translation. Using misaminoacylated tRNAs which code for suppressor tRNAs, such transcripts can be rapidly and directly detected in inexpensive kits. These kits would contain a translation system, charged suppressor tRNAs containing detectable markers, for example photocleavable coumarin-biotin, and appropriate buffers and reagents. Such a kit might also contain primers or "pre-primers," the former comprising a promoter, RBS, start codon, a region coding an affinity tag and a region complementary to the template, the latter comprising a promoter, RBS, start codon, and region coding an affinity tag - but lacking a region complementary to the template. The pre-primer permits ligation of the region complementary to the template (allowing for customization for the specific template used). A biological sample, such as diseased cells, tissue or isolated DNA or mRNA or PCR products of the DNA, is added to the

system, the system is incubated and the products analyzed. Analysis and, if desired, isolation is facilitated by a marker such as coumarin or biotin which can be specifically detected by its fluorescence using streptavidin coupled to HRP. Such kits provide a rapid, sensitive and selective non-radioactive diagnostic assay for the presence or absence of the disease.

## EXPERIMENTAL

The following examples illustrate embodiments of the invention, but should not be viewed as limiting the scope of the invention. In some of the examples below, particular reagents and methods were employed as follows:

**Reagents:** tRNA<sup>fmet</sup>, aminoacyl-tRNA synthetase, amino acids, buffer salts, and RNase free water were purchased from Sigma (St. Louis, MO). Many of the fluorescent dyes were obtained from Molecular Probes (Eugene, OR). The translation supplies including routine kits were purchased from Promega (Madison, WI). Sephadex G-25 was from Amersham-Pharmacia Biotech (Piscataway, NJ). The *in vitro* translation kits and plasmid DNAs coding for CAT (PinPoint™) and Luciferase (pBESTluc™) were from Promega (Wisconsin-Madison, WI) while DHFR plasmid DNA (pQE16-DHFR) was obtained from Qiagen (Valencia, CA). The plasmid DNA for  $\alpha$ -hemolysin, pT7-WT-H6- $\alpha$ HL was kindly supplied by Prof. Hagan Bayley (Texas A & M University) and large scale preparation of  $\alpha$ -HL DNA was carried out using Qiagen plasmid isolation kit. The bacterioopsin plasmid DNA (pKKbop) was from the laboratory stock.

**Preparation of FluoroTag tRNAs:** The purified tRNA<sup>fmet</sup> was first aminoacylated with the methionine. In typical reaction, 1500 picomoles (~1.0 OD<sub>260</sub>) of tRNA was incubated for 45 min at 37°C in aminoacylation mix using excess of aminoacyl tRNA-synthetases. After incubation, the mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in the water (25  $\mu$ l). The coupling of NHS-derivatives of fluorescent



Enzyme/Protein activities: Biological activity of  $\alpha$ -hemolysin was carried out as follows. Briefly, various aliquots (0.5-2  $\mu$ l) of *in vitro* translation reaction mixture were added to 500  $\mu$ l of TBSA (Tris-buffered saline containing 1 mg/ml BSA, pH 7.5). To this, 25  $\mu$ l of 10% solution of rabbit red blood cells (rRBCs) was added and incubated at room temperature for 20 min. After incubation, the assay mix was centrifuged for 1 min and the absorbance of supernatant was measured at 415 nm (release of hemoglobin). The equal amount of rRBCs incubated in 500  $\mu$ l of TBSA is taken as control while rRBCs incubated with 500  $\mu$ l of water as taken 100% lysis. The DHFR activity was measured spectrophotometrically. Luciferase activity was determined using luciferase assay system (Promega) and luminescence was measured using Packard Lumi-96 luminometer.

Purification of  $\alpha$ -HL and measurement BODIPY-FL incorporation into  $\alpha$ -HL: The translation of plasmid coding for  $\alpha$ -HL (His<sub>6</sub>) was carried out at 100  $\mu$ l scale and the  $\alpha$ -HL produced was purified using Talon-Sepharose (ClonTech) according to manufacturer instructions. The fluorescence incorporated into  $\alpha$ -HL was then measured on Molecular Dynamics FluorImager along with the several known concentration of free BODIPY-FL (used as standard). The amount of protein in the same sample was measured using a standard Bradford assay using Pierce Protein Assay kit (Pierce, Rockford, IL).

#### FLAG Capture Assay

##### Biotinylation of FLAG antibody

A 4.4 mg/mL stock of FLAG M2 monoclonal antibody (SIGMA Chemical, St. Louis, MO) is diluted with equal volume of 100 mM sodium bicarbonate (~15 mM final antibody concentration). Subsequently, NHS-LC-Biotin (Pierce Chemical, Rockford, IL) is added from a 2 mM stock (in DMF) to a final 150 mM. The reaction is incubated for 2 hours on ice. The mixture is then clarified by centrifugation in a microcentrifuge (14,000 R.P.M.) for 2.5 minutes. Unreacted labeling reagent is removed by gel filtration chromatography.

*Preparation of FLAG Antibody Coated ELISA Plates*

NeutrAvidin™ biotin binding protein (Pierce Chemical, Rockford, IL) is diluted to a final concentration of 50 mg/mL in 100 mM sodium bicarbonate and used to coat Microlite( 2+ white opaque 96-well ELISA plates (Dynex Technologies, Chantilly, VA). Plates are washed with TBS-T and coated using a solution of 5 mg/mL biotinylated FLAG M2 antibody in TBS-T. Plates are washed with TBS-T and blocked in Translation Dilution Buffer (TDB) [4.5% Teleostean Gelatin, 2% non-fat milk powder, 10 mM EDTA, 0.1% Tween-20, 1.25 mg/mL pre-immune mouse IgG, 2.5 mM d-biotin, in TBS, pH 7.5.].

*Binding and Detection of Target Protein*

Triple-epitope-tagged target proteins produced by *in vitro* translation using rabbit reticulocyte extract are diluted 1/25-1/75 in TDB and added to the antibody coated ELISA plates. Following capture of the target protein, plates are washed with TBS-T. Detection of c-myc is performed using a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a peroxidase labeled secondary antibody, whereas detection of the His<sub>6</sub> tag is achieved with a peroxidase labeled nickel chelate-based probe (India( His Probe-HRP, Pierce, Rockford, IL). Antibodies are diluted in TDB and the India( His Probe-HRP is diluted in TBS-T supplemented with 5 mg/mL pre-immune mouse IgG. In all cases, signal is generated using a chemiluminescent substrate system.

His-Tag Metal Affinity Capture ELISA Assay

*Binding and Detection of Target Protein*

Triple-epitope-tagged target proteins produced by *in vitro* translation using rabbit reticulocyte extract are diluted 1/25-1/75 in 1% BSA/TBS-T and added to nickel chelate coated ELISA plates (Pierce Chemical, Rockford, IL). Following capture of the target protein, plates are washed with TBS-T and blocked with 1% BSA/TBS-T. Detection of epitope tags on the bound target protein is achieved using a monoclonal FLAG M2 antibody (SIGMA Chemical, St. Louis, MO) or a polyclonal c-myc

antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in conjunction with the appropriate peroxidase labeled secondary antibody. Detection of biotin incorporated into the target protein via Biotin-lysyl-tRNA<sup>lys</sup> is achieved using NeutrAvidin™ biotin binding protein conjugated to peroxidase (Pierce Chemical, Rockford, IL). The NeutrAvidin™ conjugate and all antibodies are diluted in 1% BSA/TBS-T. In all cases, signal is generated using a chemiluminescent substrate system.

### Example 1: Preparation of Markers

Synthesis of Coumarin Amino Acid: 4-(Bromomethyl)-7-methoxy coumarin (Figure 15, compound 1; 6.18 mmole) and diethylacetamidomalonate (Figure 15, compound 2; 6.18 mmole) were added to a solution of sodium ethoxide in absolute ethanol and the mixture refluxed for 4 hours. The intermediate obtained (Figure 15, compound 3) after neutralization of the reaction mixture and chloroform extraction was further purified by crystallization from methanolic solution. This intermediate was dissolved in a mixture of acetone and HCl (1:1) and refluxed for one hour. The reaction mixture was evaporated to dryness, and the amino acid hydrochloride precipitated using acetone. This hydrochloride was converted to free amino acid (Figure 15, compound 4) by dissolving in 50% ethanol and adding pyridine to pH 4-5. The proton (<sup>1</sup>H) NMR spectrum of the free amino acid was as follows: (m.p. 274-276°C, decomp.) -OCH<sub>3</sub> (δ 3.85 s, 3H), -CH<sub>2</sub>- (δ 3.5 d, 2H), α-CH- (δ 2.9 t, 1H), CH-CO (δ 6.25 s, 1H), ring H (δ 7.05 s, 1H), (δ 7.8 d, 2H).

Synthesis of Fmoc derivative of coumarin: Coumarin amino acid (1.14 mmol) was reacted with Fluorenylmethyloxycarbonyl N-hydroxysuccinimide ester (Fmoc-NHS ester) 1.08 mmol) in the presence of 1.14 mmol of triethylamine for 30 minutes at room temperature. The reaction mixture was acidified and the precipitate washed with 1 N HCl and dried. The NMR spectrum of the free amino acid was as follows: (MP 223-225°C) -OCH<sub>3</sub> (δ 3.85 s, 3H), -CH<sub>2</sub>Br (δ 3.5 broad singlet, 2H), α-CH- (δ 3.0 t, 1H), CH-CO (δ 6.22 m, 1H), ring H (δ 7.05 s, 1H), (δ 7.8 d, 2H), fluorene H CH<sub>2</sub>-CH (δ 4.2 m, 2H), CH<sub>2</sub>-CH (δ 4.25 m, 1H), aromatic regions showed characteristic multiplets.

Synthesis of PCB: Photocleavable biotin was synthesized as described below. 2-bromo, 2'-nitroacetophenone (Figure 15, compound 5) was converted first into its hexamethyltetraammonium salt which was decomposed to obtain 2-amino, 2'-nitroacetophenone (Figure 15, compound 6). Biotin N-hydroxysuccinimidyl ester (Figure 15, compound 7; Sigma Chemical; St. Louis, MO) was reacted with a 6-aminocaproic acid (Figure 15, compound 8) to obtain the corresponding acid (Figure 15, compound 9). This acid was coupled with the 2-amino, 2'-nitroacetophenone using DCC to obtain the ketone (Figure 15, compound 10). The ketone was reduced using sodium borohydride to obtain the alcohol (Figure 15, compound 11) which was further converted into its chloroformate derivative (Figure 15, compound 12). The proton NMR spectrum of the derivative (compound 12) was as follows: ( $\delta$  1.3 m, 3H), ( $\delta$  1.4 m, 2H), ( $\delta$  1.5 m, 5H) ( $\delta$  1.62 m, 1H), ( $\delta$  2.1 t, 2H) ( $\delta$  2.4 t, 2H), ( $\delta$  2.6 d, 1H), ( $\delta$  2.8 m, 1H), ( $\delta$  3.0 t, 1H), ( $\delta$  3.1 m 1H), ( $\delta$  4.15 qt, 1H) ( $\delta$  4.42 qt, 1H), ( $\delta$  5.8 t, 1H), ( $\delta$  6.25 s, 1H), ( $\delta$  6.45 s, 1H), ( $\delta$  7.5 t, 1H), ( $\delta$  7.75 m, 4H), ( $\delta$  7.9 d, 1H).

### Example 2: Misaminoacylation of tRNA

The general strategy used for generating misaminoacylated tRNA is shown in Figure 16 and involved truncation of tRNA molecules, dinucleotide synthesis (Figure 17), aminoacylation of the dinucleotide (Figure 18) and ligase mediated coupling.

a) Truncated tRNA molecules were generated by periodate degradation in the presence of lysine and alkaline phosphatase basically as described by Neu and Heppel (J. Biol. Chem. 239:2927-34, 1964). Briefly, 4 mmoles of uncharged *E. coli* tRNA<sup>Lys</sup> molecules (Sigma Chemical; St. Louis, MO) were truncated with two successive treatments of 50 mM sodium metaperiodate and 0.5 M lysine, pH 9.0, at 60°C for 30 minutes in a total volume of 50 µl. Reaction conditions were always above 50°C and utilized a 10-fold excess of metaperiodate. Excess periodate was destroyed treatment with 5 µl of 1M glycerol. The pH of the solution was adjusted to 8.5 by adding 15 µl of Tris-HCl to a final concentration of 0.1 M. The reaction volume was increased to 150 µl by adding 100 µl of water. Alkaline phosphatase (15 µl, 30 units) was added and the reaction mixture incubated again at 60°C for two



hours. Incubation was followed by ethanol precipitation of total tRNA, ethanol washing, drying the pellet and dissolving the pellet in 20 µl water. This process was repeated twice to obtain the truncated tRNA.

b) Dinucleotide synthesis was carried out basically as performed by Hudson (J. Org. Chem. 53:617-24, 1988), and can be described as a three step process, deoxycytidine protection, adenosine protection and dinucleotide synthesis.

Deoxycytidine protection: All reaction were conducted at room temperature unless otherwise indicated. First, the 5' and 3' hydroxyl groups of deoxycytidine were protected by reacting with 4.1 equivalents of trimethylsilyl chloride for 2 hours with constant stirring. Exocyclic amine function was protected by reacting it with 1.1 equivalents of Fmoc-Cl for 3 hours. Deprotection of the 5' and 3' hydroxyl was accomplished by the addition of 0.05 equivalents of KF and incubation for 30 minutes. The resulting product (Figure 17, compound 19) was produced at an 87% yield. Phosphate groups were added by incubating this compound with 1 equivalent of bis-(2-chlorophenyl)phosphorochloridate and incubating the mixture for 2 hours at 0°C. The yield in this case was 25-30%.

Adenosine protection: Trimethylsilyl chloride (4.1 equivalents) was added to adenosine residue and incubated for 2 hours, after which, 1.1 equivalents of Fmoc-Cl introduced and incubation continued for 3 hours. The TMS groups were deprotected with 0.5 equivalents of fluoride ions as described above. The Fmoc protected adenosine (compound 22) was obtained in a 56% yield. To further protect the 2'-hydroxyl, compound 22 was reacted with 1.1 equivalents of tetraisopropyl disiloxyl chloride (TIPDSCl<sub>2</sub>) for 3 hours which produces compound 23 at a 68-70% yield. The compound was converted to compound 24 by incubation with 20 equivalents of dihydropyran and 0.33 equivalents of p-toluenesulfonic acid in dioxane for about 4-5 hours. This compound was directly converted without isolation into compound 25 (Figure 17) by the addition of 8 equivalents of tetrabutyl ammonium fluoride in a mixture of tetrahydro-furan, pyridine and water.

Dinucleotide synthesis: The protected deoxycytidine, compound 20, and the protected adenosine, compound 25 (Figure 17), were coupled by the addition of 1.1

equivalents of 2-chlorophenyl bis-(1-hydroxy benzotriazolyl) phosphate in tetrahydrofuran with constant stirring for 30 minutes. This was followed by the addition of 1.3 equivalents of protected adenosine, compound 25, in the presence of N-methylimidazole for 30 minutes. The coupling yield was about 70% and the proton NMR spectrum of the coupled product, compound 26 (Figure 17), was as follows: ( $\delta$  8.76 m, 2H), ( $\delta$  8.0 m, 3H), ( $\delta$  7.8 m, 3H) ( $\delta$  7.6 m, 4H), ( $\delta$  7.5 m, 3H), ( $\delta$  7.4 m, 18H), ( $\delta$  7.0 m, 2H), ( $\delta$  4.85 m, 14H), ( $\delta$  4.25 m, 1H); ( $\delta$  3.6 m, 2H), ( $\delta$  3.2 m, 2H) ( $\delta$  2.9 m, 3H), ( $\delta$  2.6 m, 1H), ( $\delta$  2.0-1.2 m, 7H).

c) Aminoacylation of the dinucleotide was accomplished by linking the protected marker amino acid, Fmoc-coumarin, to the dinucleotide with an ester linkage. First, the protected amino acid was activated with 6 equivalents of benzotriazol-1-yl-oxy tris-(dimethylamino) phosphonium hexafluoro phosphate and 60 equivalents of 1-hydroxybenzotriazole in tetrahydrofuran. The mixture was incubated for 20 minutes with continuous stirring. This was followed with the addition of 1 equivalent of dinucleotide in 3 equivalents N-methylimidazole, and the reaction continued at room temperature for 2 hours. Deprotection was carried out by the addition of tetramethyl guanidine and 4-nitrobenzaldoxime, and continuous stirring for another 3 hours. The reaction was completed by the addition of acetic acid and incubation, again with continuous stirring for 30 minutes at 0°C which produced the aminoacylated dinucleotide (Figure 18).

d) Ligation of the tRNA to the aminoacylated dinucleotide was performed basically as described by T.G. Heckler et al. (Tetrahedron 40: 87-94, 1984). Briefly, truncated tRNA molecules (8.6 O.D.<sub>260</sub> units/ml) and aminoacylated dinucleotides (4.6 O.D.<sub>260</sub> units/ml), were incubated with 340 units/ml T4 RNA ligase for 16 hours at 4°C. The reaction buffer included 55 mM Na-Hepes, pH 7.5, 15 mM MgCl<sub>2</sub>, 250  $\mu$ M ATP, 20  $\mu$ g/ml BSA and 10% DMSO. After incubation, the reaction mixture was diluted to a final concentration of 50 mM NaOAc, pH 4.5, containing 10 mM MgCl<sub>2</sub>. The resulting mixture was applied to a DEAE-cellulose column (1 ml), equilibrated with 50 mM NaOAc, pH 4.5, 10 mM-MgCl<sub>2</sub>, at 4°C. The column was washed with 0.25 mM NaCl to remove RNA ligase and other non-tRNA components. The tRNA--

containing fractions were pooled and loaded onto a BD-cellulose column at 4°C, that had been equilibrated with 50 mM NaOAc, pH 4.5, 10 mM MgCl<sub>2</sub>, and 1.0 M NaCl. Unreacted tRNA was removed by washes with 10 ml of the same buffer. Pure misaminoacylated tRNA was obtained by eluting the column with buffer containing 25% ethanol.

### Example 3: Preparation of Extract and Template

Preparation of extract: Wheat germ embryo extract was prepared by floatation of wheat germs to enrich for embryos using a mixture of cyclohexane and carbon tetrachloride (1:6), followed by drying overnight (about 14 hours). Floated wheat germ embryos (5 g) were ground in a mortar with 5 grams of powdered glass to obtain a fine powder. Extraction medium (Buffer I: 10 mM trisacetate buffer, pH 7.6, 1 mM magnesium acetate, 90 mM potassium acetate, and 1 mM DTT) was added to small portions until a smooth paste was obtained. The homogenate containing disrupted embryos and 25 ml of extraction medium was centrifuged twice at 23,000 x g. The extract was applied to a Sephadex G-25 fine column and eluted in Buffer II (10 mM trisacetate buffer, pH 7.6, 3 mM magnesium acetate, 50 mM potassium acetate, and 1 mM DTT). A bright yellow band migrating in void volume and was collected (S-23) as one ml fractions which were frozen in liquid nitrogen.

Preparation of template: Template DNA was prepared by linearizing plasmid pSP72-bop with EcoRI. Restricted linear template DNA was purified by phenol extraction and DNA precipitation. Large scale mRNA synthesis was carried out by *in vitro* transcription using the SP6-ribomax system (Promega; Madison, WI). Purified mRNA was denatured at 67°C for 10 minutes immediately prior to use.

### Example 4: Cell-Free Translation Reactions

The incorporation mixture (100 µl) contained 50 µl of S-23 extract, 5 mM magnesium acetate, 5 mM Tris-acetate, pH 7.6, 20 mM Hepes-KOH buffer, pH 7.5; 100 mM potassium acetate, 0.5 mM DTT, 0.375 mM GTP, 2.5 mM ATP, 10 mM creatine phosphate, 60 µg/ml creatine kinase, and 100 µg/ml mRNA containing the

genetic sequence which codes for bacterioopsin. Misaminoacylated PCB-lysine or coumarin amino acid-tRNA<sup>lys</sup> molecules were added at 170 µg/ml and concentrations of magnesium ions and ATP were optimized. The mixture was incubated at 25°C for one hour.

#### **Example 5: Isolation of Nascent Proteins Containing PCB-Lysine**

Streptavidin coated magnetic Dynabeads M-280 (Dyna; Oslo, Norway), having a binding capacity of 10 µg of biotinylated protein per mg of bead. Beads at concentrations of 2 mg/ml, were washed at least 3 times to remove stabilizing BSA. The translation mixture containing PCB-lysine incorporated into nascent protein was mixed with streptavidin coated beads and incubated at room temperature for 30 minutes. A magnetic field was applied using a magnetic particle concentrator (MPC) (Dyna; Oslo, Norway) for 0.5-1.0 minute and the supernatant removed with pipettes. The reaction mixture was washed 3 times and the magnetic beads suspended in 50 µl of water.

Photolysis was carried out in a quartz cuvette using a Black-Ray longwave UV lamp, Model B-100 (UV Products, Inc.; San Gabriel, CA). The emission peak intensity was approximately 1100 µW/cm<sup>2</sup> at 365 nm. Magnetic capture was repeated to remove the beads. Nascent proteins obtained were quantitated and yields estimated at 70-95%.

#### **Example 6: The Lower Limit of Detection using Fluorescence**

Bovine serum albumin (BSA), suspended at 0.25 mg/ml in borate buffer, pH 8.0, was combined with a 25 fold molar excess fluorescamine (Sigma Chemical; St. Louis, MO) at 50 mg/ml to produce a modified, fluorescent BSA. Various amounts of modified protein (1 ng, 5 ng, 10 ng, 25 ng, 50 ng, 75 ng, 100 ng, 150 ng, 200 ng) were suspended in loading buffer (bromophenol blue, glycerol, 2-mercaptoethanol, Tris-HCl, pH 6.8, SDS), and added to individual wells of a 1.5 mm thick, 12% polyacrylamide gel with a 3% stacker. The water cooled gel was electrophoresed for 4 hours at 50 volts. After electrophoresis, the gel was removed from the electrophoresis.

apparatus, placed on a UV transilluminator and photographed with polaroid Type 667 film using an exposure time of 10 seconds. The lowest limit of detection observed under these conditions was 10 ng. These results indicate that using equipment found in a typical molecular biology lab, fluorescently labeled proteins can be detected at ng quantities. Using even more sophisticated detection procedures and devices the level of detection can be increased even further.

#### Example 7: Nascent Proteins Containing Coumarin-Amino Acid

Cell-free translation is performed as described using charged tRNA<sup>lys</sup> molecules misaminoacylated with lysine coupled to a benzopyrene fluorophore moiety and human  $\gamma$ -interferon mRNA which contains 21 codons for lysine. Samples of the mixture are supplemented with buffer containing bromophenol blue, glycerol, 2-mercaptoethanol, Tris-HCl, pH 6.8, and SDS, and directly applied to a 12% poly-acrylamide gel (3% stacker) along with a set of molecular weight markers. Electrophoresis is performed for 3 hours at 50 volts. The gel is removed from the electrophoresis apparatus and photographed under UV light. Bands of fluorescently labeled interferon protein are specifically detected at a molecular weight of about 25 KDa. No other significant fluorescent activity is observed on the gel. Free misaminoacylated tRNA molecules may be electrophoresed off of the gel and not specifically detected.

#### Example 8: *In Vivo* Half-life of a Pharmaceutical Composition

Cell-free translation reactions are performed by mixing 10  $\mu$ l of PCB-coumarin amino acid-tRNA<sup>leu</sup>, prepared by chemical misaminoacylation as described above and suspended in TE at 1.7 mg/ml, 50  $\mu$ l of S-23 extract, 10  $\mu$ l water and 10  $\mu$ l of a solution of 50 mM magnesium acetate, 50 mM Tris-acetate, pH 7.6, 200 mM Hepes-KOH buffer, pH 7.5; 1 M potassium acetate, 5 mM DTT, 3.75 mM GTP, 25 mM ATP, 100 mM creatine phosphate and 600  $\mu$ g/ml creatine kinase. This mixture is kept on ice until the addition of 20  $\mu$ l of 500  $\mu$ g/ml human IL-2 mRNA (containing 26 leucine codons) transcribed and isolated from recombinant IL-2 cDNA. The mixture is incubated at 25°C for one hour and placed on ice. 100  $\mu$ l of streptavidin-coated

magnetic Dynabeads (2 mg/ml) are added to the mixture which is placed at room temperature for 30 minutes. After incubation, the mixture is centrifuged for 5 minutes in a microfuge at 3,000 x g or, a magnetic field is applied to the solution using a MPC. Supernatant is removed and the procedure repeated three times with TE. The  
 5 final washed pellet is resuspended in 50 µl of 50 mM Tris-HCl, pH 7.5 and transferred to a quartz cuvette. UV light from a Black-Ray longwave UV lamp is applied to the suspension for approximately 1 second. A magnetic field is applied to the solution with a MPC for 1.0 minute and the supernatant removed with a pipette. The supernatant is sterile filtered and mixed with equal volumes of sterile buffer containing  
 10 50% glycerol, 1.8% NaCl and 25 mM sodium bicarbonate. Protein concentration is determined by measuring the O.D.<sub>260</sub>.

0.25 ml of the resulting composition is injected i.v. into the tail vein of 2 Balb/c mice at concentrations of 1 mg/ml. Two control mice are also injected with a comparable volume of buffer. At various time points (0, 5 minutes, 15 minutes, 30  
 15 minutes, 60 minutes, 2 hours and 6 hours), 100 µl serum samples are obtained from foot pads and added to 400 µl of 0.9% saline. Serum sample are added to a solution of dynabeads (2 mg/ml) coated with anti-coumarin antibody and incubated at room temperature for 30 minutes. A magnetic field is applied to the solution with a MPC for 1 minute and the supernatant removed with a pipette. Fluorescence at 470 nm is  
 20 measured and the samples treated with monoclonal antibody specific for rat IL-2 protein. IL-2 protein content is quantitated for each sample and equated with the amount of fluorescence detected. From the results obtained, *in vivo* IL-2 half-life is accurately determined.

#### Example 9: Incorporation of Various Fluorophores into α-Hemolysin

25 *E. coli* tRNA<sup>fmet</sup> was first quantitatively aminoacylated with methionine and the α-amino group was specifically modified using NHS-derivatives of several fluorophores. The list of fluorescent reporter molecules (fluorophores) tested and their properties are given in Table 2. Under the modification conditions, the modified Met-tRNA<sup>fmet</sup> is found to be stable as assessed by acid-urea gel. Since all the fluorescent

molecules tested have different optical properties (excitation and emission), we have determined their relative fluorescence intensity under the condition which were used for the quantitation of gels containing nascent protein.

Fluorescent detection of nascent protein was first evaluated using  $\alpha$ -hemolysin ( $\alpha$ -HL) as a model protein (with C-terminal His<sub>6</sub>-tag).  $\alpha$ -HL is a relatively small protein (32 kDa) and could be produced efficiently in *in vitro* translation. In addition, its activity can be measured directly in the protein translation mixture using a rabbit red blood cell hemolysis assay. *In vitro* translation of  $\alpha$ -HL was carried out using an *E. coli* T7 S30 transcription/translation extract (Promega Corp., Madison, WI) in the presence of several different modified methionyl-tRNA<sup>met</sup> as described above. After the reaction, an aliquot (3-5  $\mu$ l) was subjected to SDS-PAGE analysis and the fluorescent bands were detected and quantitated using a FluorImager F595 (Molecular Dynamics, Sunnyvale, CA).

The data is presented in Figure 20. Lane 1 is a no DNA control. Lane 2 shows the results with BODIPY-FL-SSE. Lane 3 shows the results with BODIPY-FL-SE. Lane 4 shows the results with NBD (see Table 2 for the structure). Lane 5 shows the results with Bodipy-TMR. Lane 6 shows the results with BODIPY R6G. Lanes 7, 8, 9 and 10 show the results achieved with FAM, SFX, PYMPO and TAMRA, respectively (see Table 2 for structures).

The results clearly indicate the  $\alpha$ -HL produced in presence of BODIPY-FL-methionyl-tRNA<sup>met</sup> (lanes 2 and 3) exhibited the highest fluorescence (all the data is normalized to the BODIPY-FL-SSE. The two different BODIPY-FL reagents (BODIPY-FL sulfosuccinimidyl ester (SSE) and BODIPY-FL succinimidyl ester (SE)), differ only with respect to solubility. The next best fluorophore evaluated, 6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (TAMRA-X, SE), exhibited 35% of the fluorescence (corrected for relative fluorescence) of BODIPY-FL-SSE. Two other forms of BODIPY, BODIPY-TMR, SE (6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl) amino)hexanoic acid, succinimidyl ester) and BODIPY-R6G, SE (4,4-difluoro-5-phenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester)

exhibited less than 3% of the fluorescence of BODIPY-FL, SSE. Succinimidyl 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoate (NBD-X-SE), a fluorescent molecule which has previously been incorporated into the neuorkinin-2 receptor. exhibited only 6% of the BODIPY-FL-SSE. The two fluorescein analogs 5-(and-6)-carboxyfluorescein, succinimidyl-ester (FAM, SE) and 6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (SFX, SE) also showed very low fluorescence (8.4% and 4.6%, respectively relative to BODIPY-FL).

#### Example 10: Optimizing Incorporation

In order to optimize the amount of BODIPY-FL incorporated into nascent proteins, the translation reaction for  $\alpha$ -HL was carried out in presence of increasing amounts of BODIPY-FL-methionyl-tRNA<sup>fmet</sup> ranging from 3 - 60 picomoles per reaction. All reactions yielded similar amount of  $\alpha$ -HL as determined by hemolysis activity of rabbit red blood cells indicating that the exogenously added BODIPY-FL-methionyl-tRNA<sup>fmet</sup> in this range did not inhibit protein synthesis. In contrast, the intensity of the fluorescent band corresponding to  $\alpha$ -HL continued to increase up to 30 picomoles BODIPY-FL-methionyl-tRNA per 10  $\mu$ l reaction (data not shown). Increases above this level produced no further increase in fluorescence, thus subsequent reactions were performed using this level of BODIPY-FL-methionyl-tRNA.

A second step used to optimize BODIPY-FL incorporation was based on eliminating N-formyl-tetrahydrofolate (fTHF) from the reaction mixture. In prokaryotes, N-formyl-tetrahydrofolate (fTHF) acts as a cofactor for the enzyme methionyl-tRNA transformylase, which formylates the initiator tRNA after its aminoacylation with methionine. Protein synthesis is then initiated using this modified tRNA (formyl-methionine-tRNA). Without limiting the invention to any particular mechanism, it is believed that eliminating fTHF from the reaction mixture reduces the competition for initiation of protein synthesis between this endogenous initiator tRNA and exogenously added modified-initiator RNA by preventing the formylation of endogenous initiator tRNA. This was confirmed by measuring fluorescence directly



from SDS-PAGE for reactions for which fTHF was present and absent from the reaction mixture. In the later case, a 2-3 fold increase in fluorescence was found (data not shown).

### Example 11: Incorporation Into Other Proteins

5 In order to explore the general applicability of this approach, transcription/translation reactions with BODIPY-FL-methionyl-tRNA<sup>fmet</sup> were carried out using various plasmid DNAs coding for dihydrofolate reductase (DHFR), luciferase, chloramphenicol acetyl-transferase (CAT) and bacteriorhodopsin (BR). BR was included because it represents membrane proteins, which are typically very hydrophobic. An optimized coupled transcription/translation system was used along  
10 with free BODIPY-FL and BODIPY-FL-methionyl-tRNA<sup>fmet</sup> using the Talon metal chelate resin (ClonTech, Palo Alto, CA) in order to examine incorporation into other proteins. The results are shown in Figures 21A (visualization using laser based Molecular Dynamics FluorImager 595) and 21B (visualization using a UV-transilluminator). Lane 1 is a no DNA control. Lanes 2, 3, 4, 5 and 6 are hemolysin, DHFR, Luciferase, CAT and bacteriorhodopsin, respectively.  
15

Fluorescent bands are observed using a fluorescence scanner for each of the proteins at positions corresponding to their relative molecular. In the case of luciferase, bands are observed which correspond to the expected products of false  
20 initiation at internal methionines (Promega Technical Bulletin TB219). Bands corresponding to all of the proteins could also be observed visually and recorded photographically using a UV transilluminator (UVP TMW-20) combined with an emission filter that allows light with  $\lambda > 450$  nm (Figure 21B). Excitation in this case is likely occur in the UV absorbing band of BODIPY-FL which extends from 300-400  
25 nm.

The amount of BODIPY incorporation was then determined by measuring the amount of incorporated BODIPY-FL and protein present in the purified sample by comparison with solutions of different concentrations of BODIPY-FL and using a Bradford protein assay, respectively. The average of three such measurements yielded



fluorescent impurities in the gel material, extract and modified tRNA added.

### Example 13: Synthesis As A Function Of Time

The ability of the fluorescent labeling approach to monitor the nascent protein synthesis as a function of time was also evaluated. For this purpose, small aliquots of the  $\alpha$ -HL transcription/translation mixture (4  $\mu$ l) were withdrawn at various times during the reaction and analyzed by SDS-PAGE. As seen in Figure 22A, bands due to  $\alpha$ -HL can clearly be detected as early as 5 minutes after initiation of the incubation. Synthesis of fluorescently labeled  $\alpha$ -HL appears to saturate after 15 minutes of translation.

### Example 14: The Modifying Reagent

In the case of post-aminoacylation modifications used to form a misaminoacylated tRNA, an important factor is the modifying reagent used to add the modification to the natural amino acid. For example, in the case of the fluorophore BODIPY FL, there are two different commercially available BODIPY FL NHS reagents known as BODIPY-FL-SE and BODIPY-FL-SSE (Molecular Probes). Both reagents are based on N-hydroxysuccinimide (NHS) as the leaving group. However, the two forms differ in aqueous solubility due to the presence in one form (SSE) of a sulonate (sulfo) group (see Table 2 for structures). In this example, optimized reactions based on standard biochemical procedures were performed aimed at adding the BODIPY FL fluorophore to a purified tRNA<sup>met</sup> which is aminoacylated with methionine using these two different reagents. For this purpose, first the tRNA<sup>met</sup> was aminoacylated with the methionine. In typical reaction, 1500 picomoles ( $\sim 1.0$  OD<sub>260</sub>) of tRNA was incubated for 45 min at 37°C in aminoacylation mix using excess of aminoacyl tRNA-synthetases. The aminoacylation mix consisted of 20 mM imidazole-HCl buffer, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM ATP and 1600 units of aminoacyl tRNA-synthetase. The extent of aminoacylation was determined by acid-urea gel as well as using <sup>35</sup>S-methionine. After incubation, the mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to

chloroform:acid phenol (pH 5.0) extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in water (37.5 l) and used for modification.

#### A. Modification of aminoacylated tRNA with BODIPY-FL-SSE

5 To the above aminoacylated-tRNA solution, 2.5 l of 1N NaHCO<sub>3</sub> was added (final conc. 50 mM, pH = 8.5) followed by 10 l of 10 mM solution of BODIPY-FL-SSE (Molecular Probes) in water. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added  
10 and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50ml microliters of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws.

#### B. Modification of aminoacylated tRNA with BODIPY-FL-SE

15 To the above aminoacylated-tRNA solution, 2.5 l of 1N NaHCO<sub>3</sub> (final conc. 50 mM, pH = 8.5) and 20 l of DMSO was added followed by 10 l of 10 mM solution of BODIPY-FL-SE (Molecular Probes) in DMSO. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH  
20 = 5.0 was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50ml of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws.

#### 25 C. Analysis

It was found empirically using HPLC that the extent of modification of the alpha-amino group of methionine is substantially greater using the sulfonated form of

NHS BODIPY FL compared to the non-sulfonated form of NHS-BODIPY FL reagent. In addition the misaminoacylated tRNA<sup>met</sup> formed using the sulfonated form was found to exhibit superior properties. When used in an optimized S30 *E.coli* translation systems to incorporate BODIPY FL into the protein (hemolysin using a plasmid containing the HL gene under control of a T7 promoter), the band on an SDS-PAGE gel corresponding to the expressed HL exhibited an approximately 2 times higher level of fluorescence when detected using a argon laser based fluoroi-mager compared to a similar system using the misaminoacylated formed using the non-sulfonated form.

10 D. Coumarin

A similar result to that described above was obtained by comparing the non-sulfonated and sulfonated NHS derivatives of coumarin, which are also commercially available and referred to respectively as succinimidyl 7-amino-methyl-amino-coumarin acetate (AMCA-NHS; Molecular Probes) and sulfosuccinimidyl 7-amino-4-methylcoumarin-3-acetate (AMCA-sulfo-NHS; Pierce  
15 Chemicals). In this case, optimized reactions were performed using these two different reagents based on standard biochemical procedures in order to add the coumarin fluorophore to a purified tRNA<sup>met</sup> which is aminoacylated with methionine.

To the aminoacylated-tRNA solution described above, 2.5 (l of 1N NaHCO<sub>3</sub> was added (final conc. 50 mM, pH = 8.5) followed by 10 (l of 10 mM solution of sulfosuccinimidyl 7-amino-4-methylcoumarin-3-acetate (AMCA-sulfo-NHS; Pierce  
20 Chemicals) in water. The mixture was incubated for 10 min at 0(C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added and the modified  
25 tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50 microliters of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws.

To the above aminoacylated-tRNA solution, 2.5 (l of 1N NaHCO<sub>3</sub> (final conc.

50 mM, pH = 8.5) and 20  $\mu$ l of DMSO was added followed by 10  $\mu$ l of 10 mM solution of succinimidyl 7-amino-methyl-amino-coumarin acetate (AMCA-NHS; Molecular Probes) in DMSO. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50 microliter of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws.

10 In this case, the coumarin-methionine- tRNA<sup>met</sup> formed using the non-sulfonated form of coumarin-NHS (AMCA-NHS) when used in standard *E. coli* S30 translation mixtures generated very low levels of detectable fluorescent bands when detected using UV light from a standard UV transilluminator. In contrast, the sulfonated form (AMCA-sulfo-NHS) when added using the same procedures led to easily detectable bands using the UV- transilluminator.

15 Attempts to incorporate coumarin using an initiator tRNA by modifying the  $\alpha$ -amino group of methionine have been reported in the literature but failed. Coumarin attachment to a initiator tRNA subsequently required more extensive and complicated chemical attachment using a chemical cross linker. This was achieved by first aminoacylating the tRNA with methionine followed by reaction of aminoacylated tRNA with DTDG monosuccinimidyl ester (DTDG is Dithiodiglycolic acid). The reaction product was then reduced using DTT and subsequently reacted with CPM (3-(4'-Maleimidophenyl)-4-methyl-diethylamino coumarin. (Odom, O. W, Kudlicki, W and Hardestry, B. 1998. In vitro engineering using acyl-derivatized tRNA, In Protein synthesis: Methods and Protocols, PP.93-103, Humana press, Totowa, NJ.). Due to the need for special procedures designed for each marker, such an approach is not practical for general attachment of a wide variety of markers to tRNAs through post-chemical aminoacylation procedures.

25 One likely factor that makes sulfonated NHS reagents used for postchemical aminoacylation of tRNAs is its solubility in aqueous buffer. In contrast, non-sulfonated

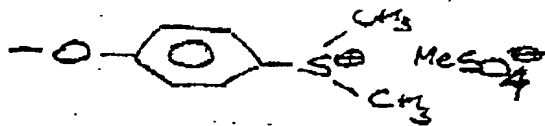
reagents such as the BIDOPY FL NHS reagent require organic buffer such as DMSO for postchemical modification. While it is still not clear why use of organic buffers lowers the overall marker incorporation, one possibility is that hydrolysis of the aminoacyl bond formed between the amino acid and tRNA reduces the overall level of modification.

### Example 15: Imparting Water Solubility

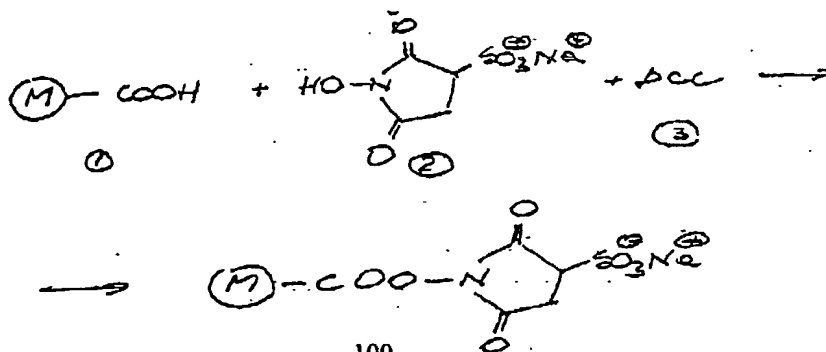
In general, the property of water solubility can be imparted to chemical reagents in several ways. Some of these are summarized below:

- Introduction of polar functional group into leaving group (such as sulfonated-NHS).
- Introduction of the polar functional group into a spacer arm.
- Introduction of the polar functional group into the reagent moiety itself.

While the introduction of the  $-\text{SO}_3^- \text{Na}^+$  (sulfo-) group is preferred, other polar ionizable groups (such as DSP) can also be used where DSP is shown below:



Final water solubility can be engineered into a spacer arm for example by using a polyether spacer (e.g. one based on tetraethylene glycol). In general, any moiety that has a free carboxyl group can be converted into its sulfo-NHS active ester. This reaction involves N-hydroxy-sulfosuccinimide (monosodium salt), the marker and a coupling agent such as DCC (dicyclohexylcarbodiimide):



In a typical reaction, marker 1, 55 mmol, is dissolved in 10 ml DMF (dimethylformamide) and (2) (5 mmol) is added, followed by (3) (1.1 equivalents). The mixture is stirred overnight at room temperature, precipitate filtered off, and the filtrate evaporated under reduced pressure at room temperature. The product is purified if necessary using column chromatography or is recrystallized.

One preferred embodiment of this invention involves the post-chemical modification of tRNAs to form a misaminoacylated tRNA by using markers that contain a sulfonated NHS reagent. While such reagents are not generally available commercially, such reagents can be routinely produced out of a variety of useful markers. For example, fluorescein, which has a high fluorescent quantum yield for both UV and visible excitation could be prepared in a form which contains a sulfonated NHS ester.

#### Example 16: Triple Marker System

In this example, a three marker system is employed to detect nascent proteins, *i.e.* an N-terminus marker, a C-terminus marker, and an affinity marker (the latter being an endogenous affinity marker). The experiment involves 1) preparation of a tRNA with a marker, so that a marker can be introduced (during translation) at the N-terminus of the protein; 2) translation of hemolysin with nucleic acid coding for wild type and mutant hemolysin; and 4) quantitation of the markers.

##### 1. Preparation of biotin-methionyl-tRNA<sup>fmet</sup>

The purified tRNA<sup>fmet</sup> (Sigma Chemicals, St. Louis, MO) was first aminoacylated with methionine. The typical aminoacylation reaction contained 1500 picomoles (-1.0 OD<sub>260</sub>) of tRNA, 20mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1mM methionine, 2 mM ATP; 150 mM NaCl and excess of aminoacyl tRNA-synthetases (Sigma). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved



in the water (25  $\mu$ l). The coupling of NHS-biotin to the  $\alpha$ -amino group of methionine was carried out in 50 mM sodium bicarbonate buffer, pH 8.0 by incubating the aminoacylated tRNA<sup>fmet</sup> (25  $\mu$ l) with NHS-biotin (final concentration = 2 mM) for 10 min at 0°C and the reaction was quenched by the addition of free lysine (final concentration = 100 mM). The modified tRNA was precipitated with ethanol and passed through Sephadex G-25 gel filtration column (0.5 x 5 cm) to remove any free reagent, if present.

## 2. *In vitro* translation of $\alpha$ -HL DNA

A WT and Amber (at position 135) mutant plasmid DNA was using coding for  $\alpha$ -hemolysin ( $\alpha$ -HL), a 32 kDa protein bearing amino acid sequence His-His-His-His-His-His (His-6) (SEQ ID NO: 5) at its C-terminal. *In vitro* translation of WT and amber mutant  $\alpha$ -HL gene (Amb 135) was carried out using *E. coli* T7 circular transcription/translation system (Promega Corp., Wisconsin, WI) in presence of Biotin-methionyl-tRNA<sup>fmet</sup> (AmberGen, Inc.). The translation reaction of 100  $\mu$ l contained 30  $\mu$ l *E. coli* extract (Promega Corp., Wisconsin, WI), 40  $\mu$ l premix without amino acids, 10  $\mu$ l amino acid mixture (1 mM), 5  $\mu$ g of plasmid DNA coding for WT and mutant  $\alpha$ -HL, 150 picomoles of biotin-methionyl-tRNA<sup>fmet</sup> and RNase-free water. The premix (1X) contains 57 mM HEPES, pH 8.2, 36 mM ammonium acetate, 210 mM potassium glutamate, 1.7 mM DTT, 4% PEG 8000, 1.25 mM ATP, 0.8 mM GTP, 0.8 mM UTP, 0.8 mM CTP, 60 mM phosphoenol pyruvate, 0.6 mM cAMP and 6 mM magnesium acetate. From the translation reaction premix, n-formyl-tetrahydrofolate (fTHF) was omitted. The translation was carried out at 37°C for 1 hour. The translation reaction mixture incubated without DNA is taken as control. After the translation reaction mixture was diluted with equal volume of TBS (Tris-buffered saline, pH 7.5). Each sample was divided into two aliquots and processed individually as described below.

## 3. Preparation of anti- $\alpha$ -HL antibody microtiter plate

Anti-rabbit-IgG coated microtiter plate (Pierce Chemicals, Rockford, Il) was

washed with Superblock buffer solution (Pierce) and incubated with 100 µg/ml of anti- $\alpha$ -HL polyclonal antibody solution (Sigma Chemicals, St. Louis, MO) prepared in Superblock buffer on microtiter plate shaker for 1 hour at room temperature. The plate was then washed (3 times x 200 µl) with Superblock buffer and stored at 4°C till further use.

4. Quantitation of N-terminal (biotin) marker

The translation reaction mixture (50 µl) for the control, WT and amber  $\alpha$ -HL DNA were incubated in different wells of anti- $\alpha$ -HL microtiter plate for 30 minutes on the shaker at room temperature. After incubation, the wells were washed 5 times (5-10 min each) with 200 µl Superblock buffer and the supernatant were discarded. To these wells, 100 µl of 1:1000 diluted streptavidin-horse radish peroxidase (Streptavidin-HRP; 0.25 mg/ml; Promega) was added and the plate was incubated at room temperature for 20 min under shaking conditions. After the incubation, excess streptavidin-HRP was removed by extensive washing with Superblock buffer (5 times x 5 min each). Finally, 200 µl of substrate for HRP (OPD in HRP buffer; Pierce) was added and the HRP activity was determined using spectrophotometer by measuring absorbance at 441 nm.

5. Quantitation of C-terminal (His-6-taq) marker

Translation reaction mixture (50 µl) from example 2 for control, WT and Amber  $\alpha$ -HL DNA were incubated in different wells of anti- $\alpha$ -HL microtiter plate for 30 min on the shaker at room temperature. After incubation, the wells were washed 5 times (5-10 min each) with 200 µl Superblock buffer and the supernatant were discarded. To these wells, 100 µl of 1:1000 diluted anti-His-6 antibody (ClonTech, Palo Alto, CA) was added to the well and incubated at room temperature for 20 min under shaking conditions. After the incubation, excess antibodies were removed with extensive washing with Superblock buffer (5 times x 5 min each). Subsequently, the wells were incubated with secondary antibody (anti-mouse IgG-HRP, Roche-BM, Indianapolis, IN) for 20 min at room temperature. After washing excess 2<sup>nd</sup>.

antibodies, HRP activity was determined as described above.

#### 6. Gel-Free Quantitation of N- and C-Terminal Markers

The results of the above-described quantitation are shown in Figures 23A (quantitation of N-terminal, Biotin marker) and Figure 23B (quantitation of C-terminal, His-6 marker). In case of *in vitro* transcription/translation of WT  $\alpha$ -HL DNA in presence of biotin-methionyl-tRNA, the protein synthesized will have translated His-6 tag at the C-terminal of the protein and some of the  $\alpha$ -HL molecules will also carry biotin at their N-terminus which has been incorporated using biotinylated-methionine-tRNA. When the total translation reaction mixture containing  $\alpha$ -HL was incubated on anti- $\alpha$ -HL antibody plate, selectively all the  $\alpha$ -HL will bind to the plate via interaction of the antibody with the endogenous affinity marker. The unbound proteins can be washed away and the N- and C-terminal of the bound protein can be quantitated using Streptavidin-HRP and anti-His-6 antibodies, respectively. In case of WT  $\alpha$ -HL, the protein will carry both the N-terminal (biotin) and C-terminal (His-6) tags and hence it will produce HRP signal in both the cases where streptavidin-HRP and secondary antibody-HRP conjugates against His-6 antibody used (HL, Figure 23A). On the other hand, in case of amber mutant  $\alpha$ -HL, only N-terminal fragment of  $\alpha$ -HL (first 134 amino acids) will be produced and will have only N-terminal marker, biotin, but will not have His-6 marker due to amber mutation at codon number 135. As a result of this mutation, the protein produced using amber  $\alpha$ -HL DNA will bind to the antibody plate but will only produce a signal in the case of streptavidin-HRP (HL-AMB, Figure 23A) and not for anti-HisX6 antibodies (HL-AMB, Figure 23B).

#### Example 17: Electrophoretic Mobility Shift Assay

To demonstrate the changes in the electrophoretic mobility of fluorescently labeled nascent protein on the SDS-gels either due to proteolysis or oligomerization in presence of membranes, we have used plasmid DNA of  $\alpha$ -hemolysin ( $\alpha$ -HL) which codes 32 kDa protein bearing a sequence His-His-His-His-His (His-6) (SEQ ID NO:5) at its C-terminal. *In vitro* translation of  $\alpha$ -HL gene was carried out using *E.*

*coli* T7 circular transcription/translation system (Promega Corp., Wisconsin-Madison, WI) in presence of BODIPY-FL-methionyl-tRNA<sup>fmet</sup> (AmberGen, Inc.) This experiment involved 1) preparation of the tRNA-marker for introduction of the N-terminus marker during translation, 2) translation, 3) purification, 4) protease treatment or 5) oligomerization.

1. Preparation of BODIPY-FL-methionyl-tRNA:

BODIPYL-FL-methionyl-tRNA was prepared by first aminoacylating pure tRNA<sup>fmet</sup> (Sigma Chemicals, St. Louis, MO) using methionine and subsequently modifying  $\alpha$ -amino group of methionine using BODIPY-FL-SSE (4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene propionic acid, sulfosuccinimidyl ester; Molecular Probes, Eugene, OR). The typical aminoacylation reaction (100  $\mu$ l) contained 1500 picomoles (-1.0 OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM methionine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl tRNA-synthetases (Sigma). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in the water (25  $\mu$ l). The coupling of BODIPY-FL-SSE to the  $\alpha$ -amino group of methionine was carried out in 50  $\mu$ l reaction volume using 50 mM sodium bicarbonate buffer, pH 8.0 by incubating 25  $\mu$ l aminoacylated tRNA<sup>fmet</sup> (1.5 nanomoles) with 10  $\mu$ l of BODIPY-FL-SSE (10 mM) for 10 min at 0°C and the reaction was quenched by the addition of free lysine (final concentration = 100 mM). The modified tRNA was precipitated with ethanol, and the pellet was dissolved in RNase-free water and passed through Sephadex G-25 gel filtration column (0.5 x 5 cm) to remove any free fluorescent reagent, if present.

2. *In vitro* translation of  $\alpha$ -hemolysin DNA

The translation reaction of 100  $\mu$ l contained 30  $\mu$ l *E. coli* extract (Promega Corp., Wisconsin, WI), 40  $\mu$ l premix without amino acids, 10  $\mu$ l amino acid mixture

(1 mM), 5  $\mu$ g of plasmid DNA coding for  $\alpha$ -HL, 150 picomoles of BODIPY-FL-methionyl-tRNA<sup>fmet</sup> and RNase free water. The premix (1X) contains 57 mM HEPES, pH 8.2, 36 mM ammonium acetate, 210 mM potassium glutamate, 1.7 mM DTT, 4% PEG 8000. 1.25 mM ATP, 0.8 mM GTP, 0.8 mM UTP, 60 mM phosphoenolpyruvate, 0.6 mM cAMP and 6 mM magnesium acetate. From the translation reaction premix, n-formyl-tetrahydrofolate (fTHF) was omitted. The translation was carried out at 37°C for 1 hour. The translation reaction mixture incubated without DNA is taken as control.

### 3. Purification of Hls-6- $\alpha$ -HL

Fifty microliters of the translation reaction mixture (from above) was subjected to Talon-Sepharose (ClonTech, Palo Alto, CA) chromatography for the purification of Hls-6- $\alpha$ -HL. This was carried out by loading the crude extract onto the Talon-Sepharose column which was pre-equilibrated with 50 mM Tris-HCl, pH 8.0 containing 150 mM NaCl and washing the column to remove unbound proteins. The bound protein was then eluted by adding 100 mM imidazole in the above buffer. The eluted  $\alpha$ -HL was dialyzed against 50 mM Tris-HCl buffer, pH 7.5.

### 4. EMSA for protease detection

The purified fluorescently labeled  $\alpha$ -HL (-5  $\mu$ g) (example 3) was incubated with 0.05  $\mu$ g of pure trypsin (Sigma Chemicals, St. Louis, MO) in 50 nM acetate buffer, pH 5.0 (100:1; protein:protease ratio) for 5 min at 37°C. The proteolysis reaction was arrested by the addition of 1X SDS-gel loading buffer and boiling the samples for 5 min. The SDS-PAGE was carried out as described by Laemmli (Laemmli, U. K. 1970, Nature 227, 680-685) using 4-20% gradient gel (ready-gel, Bio-Rad, Richmond, CA). After the gel electrophoresis, the gel was visualized using FluorImager F595 (Molecular Dynamics, Sunnyvale, CA).

Trypsin was used under very limited conditions (single-hit kinetics) to obtain very defined cleavage of  $\alpha$ -HL (50 mM acetate buffer, pH 5.0, 100:1; protein:protease ratio, 5 min at 37°C). Under these conditions, the glycine rich loop in  $\alpha$ -HL is most

susceptible to cleavage and as a result proteolytic fragment of 17 kDa was observed (Vecesey-Semjen, B., Knapp, S., Mollby, R., Goot, F.G. 1999, Biochemistry, 38 4296-4302). When the fluorescently labeled  $\alpha$ -HL was subjected to very mild trypsin treatment, it resulted a cleavage of  $\alpha$ -HL yielding the N-terminal fragment of approximately 17-18 kDa mass as evidence by change in the mobility of fluorescent band on SDS-PAGE (Figure 24: Lane 1 shows untreated protein and Lane 2 shows protease treated protein). This result indicates that such assay could be used to screen for proteases or any other enzymatic activities like kinase, transferase etc. that could potentially result in the electrophoretic mobility shift of the nascent protein. Though we have used pure nascent protein for this particular assay, there is no reason why one can not use a nascent protein without any purification (total translation reaction mixture).

#### 5. EMSA for oligomerization of nascent protein on membranes

The total translation reaction mixture (10  $\mu$ l) (see above) was incubated in absence and in presence of rabbit red blood cells (rRBCs, Charles River Farm, CT) for 30 min. at 0°C. After the incubation, rRBCs were washed free of excess unbound  $\alpha$ -HL and the rRBCs were incubated in Tris buffer saline (TBS) containing 1 mg/ml BSA (TBBSA) at 37°C for 20 min during which lysis of rRBCs occurred. The rRBC membranes were isolated after centrifugation, dissolved in 1X SDS-gel loading buffer and subjected to SDS-PAGE (4-20% gradient gel) without heating the sample. After the gel electrophoresis, the gel was visualized using FluorImager F595.

$\alpha$ -HL is expressed a soluble monomeric protein and in presence of various membranes it can oligomerize to form heptameric pore (Walker, B., Krishnasastri, M., Zorn, L., Kasianowicz, J. and Bayley, H., 1992, J. Biol. Chem. 267, 10902-10909). In addition, some intermediate forms of the oligomers were also observed. In this experiment, in order to see the applicability of EMSA to detect the shift in mobility of  $\alpha$ -HL due to oligomerization in presence lipid membranes, the total translation reaction mixture (with out any purification) was used. When the total translation extract containing nascent  $\alpha$ -HL was incubated with rRBCs, it resulted in the oligomerization.







to Laemmli (Laemmli, U.K. 1970), Nature, 227, 680-685).

#### 4. Detection of nascent protein

The gel containing nascent proteins was scanned using FluorImager F595 (Molecular Dynamics, Sunnyvale, CA using Argon laser (488 nm) as excitation source, in addition, the nascent proteins in polyacrylamide gels were also detected using an UV-transilluminator and the photographs were carried out using Polaroid camera fitted with Tiffen green filter (Polaroid, Cambridge, MA). Figures 26A and 26B show the results of *in vitro* translation of  $\alpha$ -HL produced in presence of various fluorescent tRNA<sup>lys</sup>. It is clear from the results one can incorporate a variety of fluorescent molecules into nascent protein using misaminoacylated tRNA (fluorophore-modified lysyl-tRNA<sup>lys</sup>) including dyes like NBD, fluorescein derivatives etc. (Lane 1: No DNA control; lane 2: BODIPY-FL-SSE (4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-s-indacene propionic acid, sulfosuccinimidyl ester); lane 3: BODIPY-FL-SE (4,4-difluoro-5,7-dimethyl-4bora-3a, 4a-diaza-s-indacene proionic acid, succinimidyl ester); lane 4 : NBD-X-SE (Succinimidyl 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoate); lane 5; BODIPY-TMR-SE ((6-994,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a, 4a-diaza-s-indacene-2-propionyl) amino) hexanoic acid, succinimidyl ester); lane 6: BODIPY-R6G-SE ((4,4-difluoro-5-phenyl-4-bora-3a,4a-diaza-s-indacene-3propionic acid, succinimidyl ester); lane 7; FAM-SE (5-(6-)carboxyfluorescein, succinimidyl ester); lane 8:SFX-SE (6-fluorescein-5-(and 6-)carboxyamido)hexonoic acid, succinimidyl ester); lane 9: PyMPO-SE(1-(3-(succinimidylloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl) pyridinium bromide (PyMPO)) and lane 10: TAMRA-SE (6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester).

#### Example 19: NHS-derivatives of Coumarin

Given the above noted results for the two BODIPY molecules (i.e. BODIPY-FL-SSE and BODIPY-FL-SE), attempts were made to derivatize other markers to make them suitable for incorporation. The present example involves 1) the preparation

of the labeled tRNA, 2) translation, and 3) detection of the nascent protein containing the label (or marker).

1. Preparation of Fluorescent Labeled Misaminoacylated tRNA<sup>fmet</sup>

The purified tRNA<sup>fmet</sup> (Sigma Chemicals, St. Louis, MO) was first aminoacylated with methionine. The typical aminoacylation reaction (100  $\mu$ l) contained 1500 picomoles (approximately 1.0 OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM lysine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl tRNA-synthetases (Sigma). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in the water (25 $\mu$ l). The coupling of NHS-derivatives of coumarin [namely sulfosuccinimidyl 7-amino-4-methylcoumarin-3-acetate [1] (AMCA-sulfo-NHS; Pierce Chemicals), Alexa 350-N-hydroxy-succinimide ester (Molecular Probes) and succinimidyl 7-amethyl-amino-coumarin acetate (AMCA-NHS: Molecular Probes)] to the  $\alpha$ -amino group of methionine was carried out in 50 mM sodium bicarbonate buffer, pH 8.5 by incubating the aminoacylated tRNA<sup>fmet</sup> (25  $\mu$ l) with fluorescent reagent (final concentration = 2 mM) for 10 min at 0°C and the reaction was quenched by the addition of free lysine (final concentration = 100mM). In case of AMCA-NHS, reagent was dissolved in DMSO and the coupling reaction was carried out in presence of 40% DMSO. The modified tRNA was precipitated with ethanol, dissolved in 50  $\mu$ l of RNase-free water and passed through Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws. The modification extent of the aminoacylated-tRNA was assessed by acid-urea gel electrophoresis (Varshney, U., Lee, C.P. & RajBhandary, U.L., 1991, *J. Biol. Chem.* 266, 24712-24718).

## 2. Cell Free Synthesis of Proteins in Prokaryotic (*E. coli*) Translation Extracts:

The typical translation reaction mixture (10  $\mu$ l) contained 3  $\mu$ l of *E. coli* S-30 extract (Promega Corp., Wisconsin-Madison, WI), 4  $\mu$ l of premix, 1  $\mu$ l of amino acid mix (1 mM), 30 picomoles of fluorescent-methionyl-tRNA and 0.5  $\mu$ g of  $\alpha$ -hemolysin ( $\alpha$ -HL) plasmid DNA. The premix (1X) contains 57 mM HEPES, pH 8.2, 36 mM ammonium acetate, 210 mM potassium glutamate, 1.7 mM DTT, 4% PEG 8000, 1.25 mM ATP, 0.8 mM GTP, 0.8 mM UTP, 0.8 mM CTP, 60 mM phosphoenol pyruvate, 0.6 mM cAMP and 6 mM magnesium acetate. The translation reaction was allowed to proceed for 45 min at 37°C. For SDS-PAGE, 4-10  $\mu$ l aliquot of the reaction mix was precipitated with 5-volume acetone and the precipitated proteins were collected by centrifugation. The pellet was dissolved in 1X loading buffer and subjected to SDS-PAGE after boiling for 5 min. SDS-PAGE was carried out according to Laemmli (Laemmli, U. K. 1970, Nature, 277, 680-685).

## 3. Detection of Nascent Protein

The gel containing nascent proteins was visualized using an UV-transilluminator equipped with long wavelength UV bulb (>300 nm) and the photographs were carried out using Polaroid camera fitted with Tiffen green filter (Polaroid, Cambridge, MA). Figure 27 indicates that the result *in vitro* translation of  $\alpha$ -HL produced in presence of various fluorescent tRNAs (Lane 1 shows the results for the no DNA control; Lane 2 shows the results with Met-tRNA<sup>fmet</sup> modified with AMCA-NHS; Lane 3 shows the results with Met-tRNA<sup>fmet</sup> modified with AMCA-sulfo-NHS; and Lane 4 shows the results with Met-tRNA<sup>fmet</sup> modified with Alexa-NHS). Clearly, one can incorporate the coumarin derivative molecules into nascent protein using misaminoacylated tRNA which are modified with the water soluble NHS-esters of fluorescent molecules (lane 3). Moreover, a dye with negative charge (Alexa, lane 4) seems to not incorporate as well as its neutral counterpart (AMCA; lane 3).

From the above results and general teachings of the present specification, one skilled in the art can select other markers and render them (e.g. chemically render

them) suitable for incorporation in accordance with the methods of the present invention.

### Example 20: Capillary Electrophoresis

The example describes the use of capillary electrophoresis (CE) for detection of *in vitro* synthesized fluorescent proteins by mobility shift. The example describes 1) the preparation of the tRNA comprising a BODIPY marker, 2) *in vitro* translation, 3) purification, 4) protease digestion and 5) detection by mobility shift assay.

#### 1. Preparation of BODIPY-FL-methionyl-tRNA<sup>met</sup>

The tRNA<sup>met</sup> was aminoacylated with the methionine. In typical reaction, 1500 picomoles (~1.0 OD<sub>260</sub>) of tRNA was incubated for 45 min at 37°C in aminoacylation mix using an excess of aminoacyl tRNA-synthetases. The aminoacylation mix comprised 20 mM imidazole-HCl buffer, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM ATP and 1600 units of aminoacyl tRNA-synthetase. The extent of aminoacylation was determined by acid-urea gel as well as by using <sup>35</sup>S-methionine. After incubation, the mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol (pH 5.0) extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in water (37.5 ul) and used for modification. To the above aminoacylated-tRNA solution, 2.5 ul of 1N NaHCO<sub>3</sub> was added (final conc. 50 mM, pH = 8.5) followed by 10 ul of 10 mM solution of BODIPY-FL-SSE (Molecular Probes) in water. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50 ul of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws.



## 5. Mobility Shift Assay

The SDS-capillary gel electrophoresis (SDS-CGE) was performed on a Bio-Rad BioFocus 3000CE system. The capillary was fused-silica with a 75  $\mu$ m ID, 24 cm total length and 19.5 cm to the detector. Fifty microliters of fluorescently labeled protein sample ( $\alpha$ -HL) was mixed with 50  $\mu$ l of SDS-CGE sample loading buffer and incubated at 95°C for 10 min. The capillary was rinsed with 0.1 M NaOH, 0.1 M HCl and SDS-run buffer for 60, 60 and 120 sec respectively, prior to each injection. Sample were injected using electrophoretic injection (20 sec at 10 kV). Separation was performed at 15 kV (constant voltage) for 25 min. Capillary and sample was maintained at 20°C. The detection of sample was carried out using 488 nm Argon laser and 520 nm emission filter.

The results of SDS-CGE are shown in the Figure 28. As seen in the Figure, fluorescently label  $\alpha$ -HL migrates as a major species eluting around 24 min under the electrophoresis conditions (Top panel). In addition, the electrophoregram also show the presence of minor impurities present in the sample, which are eluting around 17 and 20.5 min. When the fluorescently labeled- $\alpha$ -HL sample was treated with trypsin and analyzed using SDS-CGE, it showed peaks eluting earlier (13, 14 and 15min) and major peak at 21 min (Bottom panel). This result indicated that the  $\alpha$ -HL was proteolysed by the trypsin and various proteolytic fragments have N-terminal fluorescently labeled are seen in the electrophoregram.

### Example 21: Incorporation of Three Markers Into Hemolysin

This is an example wherein a protein is generated *in vitro* under conditions where N- and C-terminal markers are incorporated along with a marker incorporated using a misaminoacylated tRNA. The Example involves 1) PCR with primers harboring N-terminal and C-terminal detectable markers, 2) preparation of the tRNA, 3) *in vitro* translation, 4) detection of nascent protein.

1. PCR of  $\alpha$ -Hemolysin DNA

Plasmid DNA for  $\alpha$ -hemolysin, pT7-WT-H6- $\alpha$ HL, was amplified by PCR using following primers. The forward primer (HL-5) was: 5'-GAATTC-  
TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGGAAC  
5 AAAAATTAATCTCGGAAGAGGATTTGGCAGATTCTGATATTAATATTAAACC-  
3' (SEQ ID NO:11) and the reverse primer (HL-3) was: 5'-AGCTTCATTA-  
ATGATGGTGATGG-TGGTGAC 3' (SEQ ID NO:12). The underlined sequence in  
forward primer is T7 promoter, the region in bold corresponds to ribosome binding  
site (Shine-Dalgarno's sequence), the bold and underlined sequences involve the C-  
myc epitope and nucleotides shown in italics are the complimentary region of  $\alpha$ -  
10 hemolysin sequence. In the reverse primer, the underlined sequence corresponds to  
that of HisX6 epitope. The PCR reaction mixture of 100  $\mu$ l contained 100 ng template  
DNA, 0.5  $\mu$ M each primer, 1 mM  $MgCl_2$ , 50  $\mu$ l of PCR master mix (Qiagen, CA) and  
nuclease free water (Sigma Chemicals, St. Louis, MO) water. The PCR was carried  
15 out using Hybaid Omni-E thermocycler (Hybaid, Franklin, MA) fitted with hot-lid  
using following conditions: 95°C for 2 min, followed by 35 cycles consisted of 95°C  
for 1 min, 61°C for 1 min and 72°C for 2 min and the final extension at 72°C for 7  
min. The PCR product was then purified using Qiagen PCR clean-up kit (Qiagen, CA).  
The purified PCR DNA was used in the translation reaction.

20 2. Preparation of BODIPY-FL-lysyl-tRNA<sup>lys</sup>

The purified tRNA<sup>lys</sup> (Sigma Chemicals, St. Louis, MO) was first  
aminoacylated with lysine. The typical aminoacylation reaction contained 1500  
picomoles ( $\sim 1.0$  OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM  $MgCl_2$ ,  
1 mM lysine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl tRNA-synthetases  
25 (Sigma Chemicals, St. Louis, MO). The reaction mixture was incubated for 45 min at  
37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of  
3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1).  
Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained  
was dissolved in water (35  $\mu$ l). To this solution 5  $\mu$ l of 0.5 M CAPS buffer, pH 10.5

was added (50 mM final conc.) followed by 10 ul of 10 mM solution of BODIPY-FL-SSE. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50 ul of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws. The modification extent of the aminoacylated-tRNA was assessed by acid-urea gel electrophoresis. Varshney et al., *J. Biol. Chem.* 266:24712-24718 (1991).

3. Cell-free synthesis of proteins in eukaryotic (wheat germ) translation extracts.

The typical translation reaction mixture (20 ul) contained 10 ul of TnT wheat germ extract (Promega Corp., Wisconsin-Madison, WI), 0.8 ul of TnT reaction buffer, 2 ul of amino acid mix (1 mM), 0.4 ul of T7 RNA polymerase, 30 picomoles of BODIPY-FL-lysyl-tRNA<sup>lys</sup>, 1-2 ug plasmid or PCR DNA (Example 1) and RNase-free water. The translation reaction was allowed to proceed for 60 min at 30°C and reaction mixture was centrifuged for 5 min to remove insoluble material. The clarified extract was then precipitated with 5-volumes of acetone and the precipitated proteins were collected by centrifugation. The pellet was dissolved in 1X loading buffer and subjected to SDS-PAGE after boiling for 5 min. SDS-PAGE was carried out according to Laemmli, *Nature*, 227:680-685.

4. Detection of nascent protein

After the electrophoresis, gel was scanned using FluorImager 595 (Molecular Dynamics, Sunnyvale, CA) equipped with argon laser as excitation source. For visualization of BODIPY-FL labeled nascent protein, we have used 488 nm as the excitation source as it is the closest to its excitation maximum and for emission, we have used 530+/-30 filter. The gel was scanned using PMT voltage 1000 volts and either 100 or 200 micron pixel size.



The results are shown in Figure 29. It can be seen from the Figure that one can *in vitro* produce the protein from the PCR DNA containing desired marker(s) present. In the present case, the protein ( $\alpha$ -hemolysin) has a C-myc epitope at N-terminal and HisX6 epitope at C-terminal. In addition, BODIPY-FL, a fluorescent reporter molecule is incorporated into the protein. Lane 1:  $\alpha$ -Hemolysin plasmid DNA control; lane 2: no DNA control; lane 3: PCR  $\alpha$ -hemolysin DNA and lane 4: hemolysin amber 135 DNA. The top (T) and bottom (B) bands in all the lane are from the non-specific binding of fluorescent tRNA to some proteins in wheat germ extract and free fluorescent-tRNA present in the translation reaction, respectively.

### Example 22: Primer Design

It is not intended that the present invention be limited to particular primers. A variety of primers are contemplated for use in the present invention to ultimately incorporate markers in the nascent protein (as explained above). The Example involves 1) PCR with primers harboring markers, 2) *in vitro* translation, and 3) detection of nascent protein.

For PCR the following primers were used: forward primer:

**5'GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAACAAAAATTAATA**  
**TCGGAAGAGGATTTGAATGTTTCTCCATACAGGTCACGGGGA-3'** (SEQ ID

NO:13). Reverse Primer: 5'-TTATTAATGATGGTGGTGGTG-

**TTCTGTAGGAATGGTATCTCGTTTTTC-3'** (SEQ ID NO:14) The underlined sequence in the forward primer is T7 promoter, the bold and underlined sequences involve the C-myc epitope and nucleotides shown in italics are the complimentary region of  $\alpha$ -hemolysin sequence. In the reverse primer, the bold sequence corresponds to that of His-6 epitope and the underlined sequence corresponds to the complimentary region of the  $\alpha$ -hemolysin sequence. A PCR reaction mixture of 100  $\mu$ l can be used containing 100 ng template DNA, 0.5  $\mu$ M each primer, 1 mM  $MgCl_2$ , 50  $\mu$ l of PCR master mix (Qiagen, CA) and nuclease free water (Sigma Chemicals, St. Louis, MO) water. The PCR was carried out using Hybaid Omni-E thermocycler (Hybaid, Franklin, MA) fitted with hot-lid using following conditions: 95°C for 2 min, followed by 35

cycles consisted of 95°C for 1 min, 61°C for 1 min and 72°C for 2 min and the final extension at 72°C for 7 min. The PCR product can then be purified using Qiagen PCR clean-up kit (Qiagen, CA). The purified PCR DNA can then be used in a variety of translation reactions. Detection can be done as described above.

Overall, the present invention contemplates a variety of primer designs based on the particular epitopes desired (*see* Table 4 for a list of illustrative epitopes). In general, the epitopes can be inserted as the N-terminus or C-terminus. In addition, they can be used to introduce an affinity region (*i.e.* a region which will bind to antibody or other ligand) into the protein.

### Example 23: Antibody Detection of Primer-Encoded Epitopes

This is an example wherein a protein is generated *in vitro* under conditions where affinity regions are incorporated in a protein and thereafter detected. The Example involves 1) PCR with primers containing sequences that encode epitopes, 2) preparation of the tRNA, 3) *in vitro* translation, 4) detection of nascent protein.

#### 1. PCR with Primer-encoded epitopes

The total RNA from the human colon (Clontech, Palo Alto, CA) was subjected to one-step RT-PCR reaction using ClonTech RT-PCR Kit. The forward Primer, PTT-T7-P53, was [5'-GGATCCTAATACGACTCACTATAGGGAGACCACCA-TGGGACACCACCATCACCATCACGGAGATTACAAAGATGACGATGACAAA-GAGGAGCCGCAGTCAGATCCTAGCGTCGA-3'] (SEQ ID NO:15) and the reverse primer, Myc-P53-3', was [5'-ATTATTACAAATCCTCTTCCGAGATTAATT-TTTGTTCGTCTGAGTCAGGCCCTTCTGTCTTGAACATG-3'] (SEQ ID NO:16). The underlined sequence in forward primer is T7 promoter, the nucleotides shown in italics corresponds to that of His-6 tag while the sequence in bold codes for FLAG-epitope and the rest of primer is the complementary region for P53 DNA. In the reverse primer, the underlined sequence corresponds to that of c-Myc epitope.

The RT-PCR/PCR reaction mixture of 50µl contained 1µg total human colon RNA, 0.5µM each primer, 43.5µl of RT-PCR master mix (ClonTech) and nuclease...

free water (Sigma Chemicals, St. Louis, MO) water. The RT-PCR/PCR was carried out in PTC-150 thermocycler (MJ Research, Waltham, MA) using following conditions: 50°C for 1 hour, 95°C for 5 min followed by 40 cycles consisted of 95°C for 45 sec, 60°C for 1 min and 70°C for 2 min and the final extension at 70°C for 7 min. The PCR product was analyzed on 1% agarose gel and the PCR amplified DNA was used in the translation reaction without any further purification. The artificial C-terminal truncated mutant of P53 was prepared using the identical procedure described above except the reverse primer, 3'-P53-Mut, was 5'-CTCATTCAGCTCTCGGAACATC-TCGAAGCG-3' (SEQ ID NO:17).

## 2. tRNA labelling

Purified tRNA<sup>lys</sup> (Sigma Chemicals, St. Louis, MO) was first amino-acylated with lysine. The typical aminoacylation reaction (100µl) contained 1500 picomoles (-1.0 OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM lysine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl tRNA-synthetases (Sigma). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in the water (35µl). To this solution 5ul of 0.5M CAPS buffer, pH 10.5 was added (final concentration of 50 mM) followed by 10 ul of 10 mM solution of BODIPY-FL-SSE. The mixture was incubated for 10 minutes at 0°C and the reaction was quenched by the addition of free lysine (final concentration = 100 mM). To the resulting solution 0.1 volumes of 3 M NAOAc (pH=5.0) was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50µl of RNase-free water and passed through Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid freeze-thaws. The modification extent of the aminoacylated-tRNA was assessed by acid-urea gel electrophoresis [Varshney, U., Lee, C.P. & RajBhandary, U.L., *J. Biol. Chem.* **266**, 24712-24718 (1991)] or by HPLC [*Anal. Biochem.*

279:218-225 (2000)].

### 3. Translation

Translation of P53 DNA (*see* step 1, above) was carried out in rabbit reticulocyte translation extract in presence of fluorescent-tRNA (step 2, above).

### 4. Detection

Once the translation was over, an aliquot (5 $\mu$ l) was subjected to SDS-PAGE and the nascent proteins were visualized using FluorImager SI (Molecular Dynamics, Sunnyvale, CA). After visualization, the gel was soaked in the transfer buffer (12 mM Tris, 100 mM glycine and 0.01 % SDS, pH 8.5) for 10 min. Proteins from the gels were then transferred to PVDF membrane by standard western blotting protocol using Bio-Rad submersion transfer unit for 1 hr. After the transfer, then membrane was reversibly stained using Ferrozine/ferrous total protein stain for 1 min to check the quality of transfer and then the membrane was blocked using amber blocking solution (4.5% v/v teleostean gelatin, 2% w/v non-fat milk powder, 0.1% w/v Tween-20 in Tris-buffered saline, pH 7.5) for 2 hours followed by overnight incubation (12-15 hours at 4°C on constant speed shaker) with appropriately diluted antibodies. For Flag detection, we have used 2000-fold diluted anti-Flag M2 Antibody (Sigma), for His-6 detection, we have used 500-fold anti-His6 antibody (Santa-Cruz Biotech, CA) and for c-Myc detection, we have used 500-fold diluted anti-C-Myc antibody (Santa-Cruz Biotech, CA).

After primary antibody incubation, the membrane was washed with TBST (Tris-buffered saline, pH 7.5 with 0.1% Tween-20) four times (10 min each wash) and incubated with appropriately diluted secondary antibodies (10,000-fold diluted) for 1 hour at room temperature on constant speed shaker. The unbound secondary antibodies were washed with TBST (4 washes/ 10 min each) and the blot was visualized using an ECL-Plus chemiluminescence detection system (Amersham-Pharmacia Biotech, NJ).

The results are shown in Figure 30A and 30 B. Figure 30A shows the total protein stain of PVDF membranes following protein-transfer from the gel for three

replicate blots containing a minus DNA negative control and a plus p53 DNA sample respectively. Figure 30B shows the same blots (total protein staining is reversible) are probed with antibodies against the three epitope tags using standard chemiluminescent Western blotting techniques. Arrows indicate the position of p53.

#### Example 24: Gel-based PTT For Cancer Genes

The detection of truncating mutations in proteins was first reported by Roest and co-workers and applied to the detection of truncating mutations in the APC gene by Vogelstein, Kinzler and co-workers. Truncations in a translated protein can occur due to frameshift, splicing and point mutations which result in the occurrence of a stop codon in the reading frame of a gene. Truncated polypeptides can be detected by translating a specific region of the DNA corresponding to the target gene in an *in vitro* system in the presence of radioactive labels (e.g. <sup>35</sup>S-methionine) and then analyzing the resulting polypeptide using standard PAGE. Such an approach has been reported for the analysis of truncating mutations in a variety of cancer-linked genes including BRCA1/BRCA2, ATM, MHS2, MLH1. However, the use of radioactive isotopes presents problems in terms of the time needed for detection (>5 hours), which is critical for high-throughput analysis. For this reason, it would be highly advantageous to replace radioactivity with a more rapid means of detection.

In this example, we demonstrate the feasibility of rapid truncation analysis based on the use of N-terminal tags. The present invention provides a convenient, accurate and rapid method to screen for truncation mutations in a wide range of genes of clinical significance. The Example involves 1) PCR with primers having sequences complementary to the APC gene, 2) preparation of the tRNA, 3) *in vitro* translation, 4) detection of nascent protein.

##### 1. PCR of clinical samples

Clinical samples were submitted to the Washington University Molecular Diagnostics laboratory for screening of chain truncations in the APC gene, which are characteristic of the autosomal dominant cancer syndrome familial adenomatous

polyposis (FAP). Genomic DNA was isolated and a specific region of the APC gene (Exon 15-segment 2) was first amplified by PCR using primers which incorporate a T7 promoter, and Kozak sequence into the DNA. The forward Primer, T7-APC2 was [5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGATGCATGTGGA-  
 5 ACTTTGTGG-3'] (SEQ ID NO:18) and the reverse primer 3'-APC2 was [5'-GAGGATCCATTAGAT-GAAGGTGTGGACG-3'] (SEQ ID NO:19). The underlined sequence in forward primer is T7 promoter and the sequence shown in italics corresponds to that of Kozak sequence which is necessary for efficient eukaryotic translation initiation. The PCR reaction mixture of 50µl contained 200-500  
 10 ng template DNA (either WT or mutant), 0.5µM each primer and 25µl of PCR master mix (Qiagen, CA) and nuclease free water (Sigma Chemicals, St. Louis, MO) water. The PCR was carried out using Hybaid Omni-E thermocycler (Hybaid, Franklin, MA) fitted with hot-lid following conditions: 95°C for 3 min, followed by 40 cycles consisted of 95°C for 45 sec. 55°C for 1min and 72°C for 2 min and the final  
 15 extension at 72°C for 7 min. The PCR product was analyzed on 1% agarose gel and the PCR amplified DNA was used in the translation reaction without any further purification.

## 2. Preparation of the tRNA

The purified tRNA<sup>lys</sup> (Sigma Chemicals, St. Louis, MO) was first amino-  
 20 acylated with lysine. The typical aminoacylation reaction (100µl) contained 1500 picomoles (-1.0 OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM lysine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl tRNA-synthetases (Sigma). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium  
 25 acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in the water (35µl). To this solution 5ul of 0.5M CAPS buffer, pH 10.5 was added (final concentration of 50 mM) followed by 10 ul of 10 mM solution of BODIPY-FL-SSE. The mixture was incubating for 10 minutes at 0°C and the reaction was

quenched by the addition of free lysine (final concentration = 100 mM). To the resulting solution 0.1 volumes of 3 M NAOAc (pH=5.0) was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50µl of RNase-free water and passed through Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws. The modification extent of the aminoacylated-tRNA was assessed by acid-urea gel electrophoresis (Varshney, U., Lee, C.P. & RajBhandary, U.L., 1991 J. Biol. Chem. 266, 24712-24718).

### 3. Translation

The PCR products (see step 1 above) were directly added without purification to a small aliquot of a Promega rabbit reticulocyte TnT Quick system which also contained the BODIPY-Lys-tRNA (see step 2 above). More specifically, after PCR, 0.5 - 1µl of PCR product was directly added to translation reaction mixture containing 8µl of rabbit reticulocyte extract for PCR product (Promega), 0.5µl of 1 mM complete amino acid mix, 1µl of BODIPY-FL-Lysyl-tRNA. The translation reaction was allowed to proceed for 1 hour and the reaction product were analyzed by 14% SDS-PAGE. Imaging was performed in under 1-2 minute using a Molecular Dynamics FluorImager.

### 4. Detection

Figure 31 shows the results for analysis of several different human genomic samples using BODIPY-FL-lysyl-tRNA<sup>lys</sup> to incorporate a fluorescent label into fragments of the APC protein. Lane 1 is a minus DNA control. Lane 2 shows the results for wild-type DNA, while lanes 3-8 show the results for various mutant DNA isolated from patients having FAP (colon cancer). The last lane is fluorescent molecular weight markers. As seen in Figure 31, the WT DNA (lane 2) produces a band, which corresponds to the normal Exon 15, segment 2 fragment of the APC gene. In contrast, all other lanes (except lane 6) exhibit the WT band *and an additional band* which corresponds to truncated fragments of Exon 15, segment 2. Thus, these

individuals are heterozygous and carry one WT and one chain truncating mutation in the APC gene. In contrast, the lane 6 results indicates normal WT sequence in this region for both genes. Similar conclusion was reached independently using conventional radioactive PAGE analysis of patient samples by the University Molecular Diagnostics laboratory.

A similar analysis was performed to detect chain-truncating mutations in Exon 15-segment 3 (Figure 32). Proteins were synthesized using the rabbit reticulocyte *in vitro* translation system in conjunction with BODIPY-FL-lysyl-tRNA<sup>lys</sup>. Following separation by SDS-PAGE, translated proteins were visualized by fluorescence imaging (Figure 32A) or by chemiluminescent Western blotting procedures using a polyclonal antibody directed against the BODIPY fluorophore (Figure 32B). Lane 1 is a minus DNA control. Lane 2 shows the results for APC3 wild-type DNA, while lane 3 shows the results for APC3 truncated mutant. Lane 4 shows the results for APC2 wild-type DNA, while lane 5 shows the results for APC2 heterozygous mutant.

Overall, these results demonstrate the ability to replace radioactive PTT screening with fluorescent-based screening of chain truncations involved in human inherited diseases.

#### Example 25: Gel-free PTT For Cancer Genes

Although the replacement of radioactivity in Example 24 (above) with fluorescent labels represents an improvement in current PTT technology, it still relies on the use of gels, which are not easily adaptable for high-throughput screening applications. For this reason, this example demonstrates a non-gel approach based on the use of chemiluminescent detection. In this approach, a cancer-linked protein or polypeptide fragment from the protein is expressed *in vitro* from the corresponding gene with different detection and binding tags incorporated at the N-terminal, C-terminal and between the two ends of the protein using a combination of specially designed primers and tRNAs. The detection and binding tags provide a means to quantitate the fraction of protein or protein fragment which is truncated while the tags located between the two ends of the protein can be used to determine the region of



truncation. For example, a full-length protein would contain both an N and C-terminal tag, whereas a truncated protein would contain only the N-terminal tag. The signal from a tag incorporated at random lysines between the two ends of the protein (intrachain signal) would be reduced proportional to the size of the truncated fragment. It is important to also capture the protein with a marker located close to the N-terminus in order to avoid interference of chain truncations with binding.

In order to evaluate this method, we performed experiments on the APC and p53 genes containing either a WT sequence or truncating mutations. In both cases, a combination of primers and specially designed tRNAs were used to incorporate a series of markers into the target proteins during their *in vitro* synthesis in a rabbit reticulocyte system. After *in vitro* expression, the expressed protein was captured in 96-well ELISA plates using an affinity element bound to the plate. The relative amount of N-terminal, C-terminal and intrachain signal was then determined using separate chemiluminescent-based assays.

# 1. PCR of Cancer Genes

## A. APC segment 3

First, the genomic DNA (WT and isolated from cell lines harboring mutant APC gene) was amplified by PCR using following primers. The forward primer, PTT-T7-APC3, was [5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATG-CACCACCATCACCATCACGGAGGAGATTACAAAGATGACGATGACAAA-GTTTCTCCATACAGGTCACGGGGAGCCAAT-3'] (SEQ ID NO:20) and the reverse primer, PTT-Myc-APC3, was [5'-ATTATTACAAATCCTCTTCCGAGATTAA-TTTTGTTCACCTTCTGCCTTCTGTAGGAATGGTATCTCG-3'] (SEQ ID NO:21).

The underlined sequence in forward primer is T7 promoter, nucleotides shown in italics corresponds to that of His-6 tag while the nucleotides sequence shown in the bold codes for FLAG-epitope and the rest of the primer is the complementary region for APC segment 3 DNA. In the reverse primer, the underlined sequence corresponds to that of c-Myc epitope. The PCR reaction mixture of 50µl contained 200-500 ng template DNA (either WT or mutant), 0.5µM each primer and 25µl of PCR master

mix (Qiagen, CA) and nuclease free water (Sigma Chemicals, St. Louis, MO) water. The PCR was carried out using Hybaid Omni-E thermocycler (Hybaid, Franklin, MA) fitted with hot-lid using following conditions: 95°C for 3 min, followed by 40 cycles consisting of 95°C for 45 sec, 55°C for 1 min and 72°C for 2 min and the final extension at 72°C for 7 min. The PCR product was analyzed on 1 % agarose gel and the PCR amplified DNA was used in the translation reaction without any further purification.

#### B. P53

The p53 DNA was prepared as described in Example 23 (above).

#### 2. Preparation of the tRNA

The BODIPY-FL-lysyl-tRNA<sup>lys</sup> was prepared as described in Example 23 (above). Preparation of Biotin-lysyl-tRNA<sup>lys</sup> and PC-Biotin-lysyl-tRNA<sup>lys</sup> was achieved as follows. The purified tRNA<sup>lys</sup> (Sigma Chemicals, St. Louis, MO) was first aminoacylated with lysine. The typical aminoacylation reaction contained 1500 picomoles (-1.0 OD<sub>260</sub>) of tRNA, 20 mM imidazole-HCl buffer, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM lysine, 2 mM ATP, 150 mM NaCl and excess of aminoacyl-tRNA-synthetases (Sigma Chemicals, St. Louis, MO). The reaction mixture was incubated for 45 min at 37°C. After incubation, the reaction mixture was neutralized by adding 0.1 volume of 3 M sodium acetate, pH 5.0 and subjected to chloroform:acid phenol extraction (1:1). Ethanol (2.5 volumes) was added to the aqueous phase and the tRNA pellet obtained was dissolved in water (35 µl). To this solution 5 µl of 0.5 M CAPS buffer, pH 10.5 was added (50 mM final conc.) followed by 10 µl of 10 mM solution of either Biotin or photocleavable-Biotin. The mixture was incubated for 10 min at 0°C and the reaction was quenched by the addition of lysine (final concentration = 100 mM). To the resulting solution 0.1 volume of 3 M NaOAc, pH = 5.0 was added and the modified tRNA was precipitated with 3 volumes of ethanol. Precipitate was dissolved in 50 µl of water and purified on Sephadex G-25 gel filtration column (0.5 X 5 cm) to remove any free fluorescent reagent, if present. The modified

tRNA was stored frozen (-70°C) in small aliquots in order to avoid free-thaws. The modification extent of the aminoacylated-tRNA was assessed by acid-urea gel electrophoresis (Varshney, U., Lee, C.P. & RajBhandary, U.L., 1991, J. Biol. Chem. 266, 24712-24718).

5        3.        Translation

The typical translation reaction mixture (20µl) contained 16µl of TNT rabbit reticulocyte extract for PCR DNA (Promega, Madison, WI), 1µl of amino acid mix (1 mM), 1-2µl of PCR DNA (*see* APC and p53 preparation described above) and RNase-free water. For fluorescence detection, the BODIPY-FL-lysyl-tRNA<sup>lys</sup> was  
10 included into the translation reaction mixture. The translation reaction was allowed to proceed for 60 min at 30°C.

4.        Detection

Figure 33A shows the results of an initial experiment designed to detect a chain truncation introduced into the p53 protein during RT-PCR. In this case an N-terminal  
15 FLAG epitope was used for capture (*see* the description of the capture assay using 96-well ELISA plates at the beginning of the EXPERIMENTAL section), and His<sub>6</sub> and c-myc used for the N- and C-terminal markers, respectively. Detection of the N-terminus His-tag was achieved using a peroxidase labeled nickel chelate-based detection probe (India<sup>TM</sup> His Probe-HRP, Pierce, Rockford, IL). Detection of the C-  
20 terminus was performed using a rabbit polyclonal antibody directed against the human c-myc epitope followed by a peroxidase labeled mouse anti-[rabbit IgG] secondary antibody. As seen, the ratio of C/N terminal signals is reduced approximately 25-fold for the truncated protein compared to WT. Further optimization of this assay should result in sensitivity sufficient to detect truncating mutations in 1/100 mutant/WT p53  
25 proteins, thus enabling applications to non-invasive colon cancer screening.

In a second experiment (Figure 33B), capture was facilitated with an N-terminal His<sub>6</sub>-tag, while FLAG and c-myc were used as N and C-terminal markers, respectively. In addition, an intrachain photocleavable biotin marker was incorporated



The SDS-PAGE was carried out according to Laemmli (Laemmli, U. K. 1970, Nature, 227, 680-685).

The gel was then scanned using FluorImager SI (Molecular Dynamics, Sunnyvale, CA) equipped with argon laser as excitation source. For visualization of BODIPY-FL labeled nascent protein, 488 nm was used as excitation source as it is the closest to its excitation maximum and for emission (we have used 530±30 filter). The gel was scanned using PMT voltage 1000 volts and either 100 or 200 micron pixel size.

Figure 34 shows the results. Lane M contains fluorescent markers. Lane 1 is a minus DNA control (+ anti-HL antibody). Lane 2 shows the results for HL DNA + mouse IgG. Lane 3 shows the results for HL DNA + anti-HL antibody. Lane 4 is a minus DNA control (+ anti-P53 antibody). Lane 5 shows the results for p53 DNA + mouse IgG. Lane 6 shows the results for p53 DNA + anti-p53 antibody. It should be clear that the immunoprecipitation method provides specific results and a very clean signal without any substantial background.

#### Example 26: Suppressor tRNAs

When using BODIPY-labeled initiator tRNAs, a competition occurs between endogenous initiator tRNA (unlabeled) which causes reduced incorporation of the label. To overcome this competition, the present invention contemplates using an amber stop codon and a corresponding suppressor tRNA.

##### 1. Preparation of BODIPY-Val-pdCpA

The first step involves the preparation of a BODIPY-amino acid conjugate for attachment to the suppressor tRNA. The synthesis is set forth in Figure 35. The synthesis begins with the preparation of 5-Methyl-1(2-Nitrophenyl)ethanol (compound 2). To begin, 5-Methyl-2-nitroacetophenone (Olejnik, J., S. Sonar, E. Krzymanska-Olejnik, and K. J. Rothschild. 1995 *Proc Natl Acad Sci U S A*. 92:7590-4; Olejnik, J., E. Krzymanska-Olejnik, and K. J. Rothschild. 1998. *Methods Enzymol.* 291:135-54.) was dissolved in 5 ml of ethanol. To this solution 5 mg of

sodium borohydride was added in small portions until analytical TLC showed complete conversion to 5-Methyl-1(2-Nitrophenyl)ethanol. The reaction was quenched by addition of 5 ml acetone and acidified to pH = 3.0 with 1 N HCl, followed by extraction with chloroform. The organic layer was dried and evaporated to give the target compound as yellowish oil. This compound was used without any further purification.

Continuing with the synthesis scheme, 5-Methyl-1(2-Nitrophenyl)ethanol (350 mg; 1.95 mmol) (compound 2) was dissolved in 10 ml of acetonitrile. To this solution 407 ul of triethylamine (1.5 eq) was added followed by N,N'-disuccinimidyl carbonate (750 mg, 1.5 eq.). The reaction mixture was stirred at room temperature until analytical TLC (silica gel, chloroform) showed quantitative conversion to compound 3. The resulting solution was added directly to the solution of triethylammonium salt of valine.

Valine (218 mg; 1.86 mmol) was suspended in 2 ml of water to which was added 260 ul of triethylamine. To this solution a solution of compound 3 was added over 15 min. period, during which pH value of the mixture was adjusted if necessary by the addition of triethylamine. The mixture was stirred for 30 min and acidified by addition of 10 ml of 0.1 N HCl, followed by extraction with 3 x 20 ml AcOEt. Organic layers were combined, dried and evaporated to dryness. The crude product was dissolved in 20 ml chloroform and extracted with 4 x 30 ml of 0.1 N NaHCO<sub>3</sub>, the aqueous layer acidified and re-extracted by chloroform to give 320 mg of pure target Npe-Val (compound 4).

Npe-Val (compound 4) (320 mg, 1 mmol) was dissolved in 320 ul of acetonitrile. To this solution was added triethylamine (280 ul, 2 eq.) and chloroacetonitrile (190 ul, 3 eq.). The mixture was stirred overnight at room temperature. 20 ml of methylene chloride was added followed by 20 ml of 1N NaHSO<sub>4</sub>, the mixture was extracted with 4 x 20 ml methylene chloride. Organics were evaporated to dryness and purified on silica gel column using step gradient of ethyl acetate in hexane (10-50%) to give 300 mg of pure product (compound 5).

20 OD of pdCpA (Annovis) containing 2.2 equiv of tetrabutylammonium

counterion was dissolved in 40 ul of dry DMF. To this solution was added a solution of 2 equivalents of Npe-Val-COOCH<sub>2</sub>CN (0.66 mg) in 10 ul DMF. The reaction was kept at RT for 2 hrs, then the product was isolated using preparative HPLC to give 15 OD of compound 6.

5 15 OD<sub>260</sub> of Npe-Val-pdCpA was dissolved in 500 ul of 25 mM NaOAc, pH = 4.5 and irradiated for 20 minutes using 365 nm light (BlakRay XX-15, UVP). 166 ul of BODIPY-FL-SE (@10mg/ml; 7 equivs. was added) followed by 140 ul of 1N NaHCO<sub>3</sub>. Additional portions of BODIPY-FL-SE solution (2 x 166 ul, total 21 equivs) were added after 5 and 10 minutes, respectively, and the reaction mixture was vortexed  
10 at room temperature for 45 minutes. The product (compound 7) was isolated using preparative HPLC Novapak C18, 10x100mm, linear gradient of acetonitrile in 50 mM TEAA, pH 5.4 over 90 min., flow 2 ml/min) and characterized by absorption at both 260 and 505 nm. Yield 7.25 OD<sub>260</sub>.

2. Expression and purification of amber-suppressor fMet-tRNA.

15 PstI fragment of *nusA* operon containing a gene for mutant tRNA<sup>fmet</sup> (S.Ishi et al. PNAS v.81, pp409-413, 1984) was cloned into pGEM4 vector (Promega, Madison, WI). Total tRNA was isolated from overnight culture (100 ml) of *E. coli* XL-blue 2 cells by phenol extraction and ethanol precipitation. Suppressor tRNA<sup>fmet</sup> was purified using native PAGE (Seong and RajBhandary, PNAS v.84 pp.334-338,  
20 1987).

3. tRNA truncation by venom phosphodiesterase I (VPD I) (Figure 36)

1 OD<sub>260</sub> of purified initiator suppressor tRNA was dissolved in 50 uL of 50 mM Tris-HCl pH 7.5; 10 mM MgCl<sub>2</sub> and 100 mM NaCl. To this solution 0.5 unit of VPD I was added and the mixture was incubated for 30 min at 0°C. After phenol  
25 extraction tRNA was precipitated by EtOH, dried and dissolved in 100 uL of water. Selective (-CA) dinucleotide removal from the 3'-terminus was confirmed by 8%, 7M Urea PAGE analysis.

4. Ligation with BODIPY-Val-pdCpA with truncated initiator suppressor tRNA

As shown in the scheme of Figure 36, the tRNA is charged with the BODIPY-amino acid conjugate in a ligation reaction. 0.5 OD<sub>260</sub> of tRNA was incubated with 0.5 OD<sub>260</sub> unit of pdCpA in 100 ul of ligation buffer (50 mM Tris-HCl pH 7.8; 10 mM MgCl<sub>2</sub>; 10 mM DTT; 1 mM ATP 30% of DMSO) and 200 units of T4 RNA ligase (New England Biolabs, Beverly, MA) for 1 hour at 37°C. After the incubation tRNA was precipitated by 3-4 volumes of EtOH, dissolved in 50ul of water and spine-filtered using G25 MicroSpin column (Amersham Pharmacia Biotech Inc.). Ligated tRNA was aliquoted and stored at -70°C. The product will be referred to as an initiator/amber suppressor tRNA.

5. Translation and analysis

Translation was performed in a cell-free *E. coli* S30 extract (Promega, Madison, WI) in 20 ul total volume. The mixture contained DNA coding for the particular protein (1 ug) and initiator amber suppressor tRNA prepared by chemical aminoacylation (1 ug). The mixture was incubated for 1 hour at 37 °C and then 1 ul was mixed with the loading buffer, heat denatured and run on a 14% SDS-PAGE (150 V, 1 hr). The gel was then rinsed briefly and analyzed using Fluoroimager in case of BODIPY label. In case of PC-Biotin the gel was blotted onto PVDF membrane and the biotin detected using streptavidin-HRP.

Figure 37 shows the results. Lane 1 is a minus DNA control + BODIPY-Val initiator/amber suppressor tRNA. Lane 2 shows the results for luciferase DNA + BODIPY-Lys-tRNA. Lane 3 shows the results for amber-1 luciferase DNA + BODIPY-Lys-tRNA, while lane 4 shows the improved results for amber-1 luciferase DNA + BODIPY-Val initiator/amber suppressor tRNA.

Lane 5 shows the results for hemolysin DNA + BODIPY-Lys-tRNA. Lane 6 shows the results for amber-1 hemolysin DNA + BODIPY-Lys-tRNA, while lane 7 shows the improved results with amber-1 hemolysin DNA + BODIPY-Val initiator/amber suppressor tRNA.

Lane 8 shows the results for DHFR DNA + BODIPY-Lys tRNA. Lane 9







5 rabbit reticulocyte this would cost approximately \$800,000. In contrast, smaller volume 50 microliter reactions compatible with 2-d microarray formats would cost only \$20,000. Earlier methods of *in vitro* expression cloning such as described in United States Patent 5,654,150 (hereby incorporated by reference) are based on collecting  
10 pools of about 100 individual bacterial colonies and expressing proteins encoded by the cDNAs in the pools *in vitro*. While this approach represents an improvement of earlier (non-*in vitro*) expression cloning methods such as described in U.S. Pat. No. 4,675,285 (also incorporated by reference), it still does not generally provide a sufficiently sensitive method to perform high throughput drug-proteomic screening  
15 provided by the present invention. In addition to drug-proteomic screening, the present invention would also allow for the rapid throughput screening of interactions of the proteome with DNA and other biomolecules.

Two different related approaches for drug-proteomic screening are contemplated, both based on the utilization of 2-D arrays or microarrays and detection  
20 using fluorescence, luminescence, chemiluminescence, absorption or other means of electromagnetic detection (either directly or indirectly). As shown in Figure 38, the first approach utilizes cell-free expressed proteins produced from pools of plasmids, which comprise a formatted genomic library. In order to eliminate interference from preexisting protein in the reaction mixture, which exist at much higher levels than the  
25 expressed proteins, a pre-purification step may be necessary using incorporated affinity tags and complementary capture molecules coated on a surface. Each sample from an individual pool containing the expressed proteins is then applied to a spot on the surface containing the immobilized target drug. The incorporated fluorescence marker is then used to identify those samples, which contain proteins, which interact with the  
30 target drug. Alternatively, a fluorescently labeled molecule (*e.g.* an antibody), which interacts with an incorporated affinity marker can be used. In one preferred embodiment, a biotin is used as the affinity marker, which interacts with a streptavidin-horse radish peroxidase (HRP) complex which catalyzes a reaction that produces a detectable signal (*e.g.* chemiluminescent or fluorescent).

Once a protein pool is identified which exhibits an interaction with the target

drug through detection of fluorescence from the particular spot, the actual interacting protein can be identified using a variety of procedures. One method relies on the retransformation of the individual plasmids in the pool followed by *in vitro* expression of proteins from each individual plasmids in the presence of FluoroTags or Affinity Tags. An alternative approach is to recover the actual bound protein(s) from a spot showing positive fluorescence and perform sequence analysis either using mass spectrometry or through chemical means.

A second related approach for drug-proteome screening is also contemplated (Figure 39). In this case, the samples containing the cell-free expressed proteins produced from pools of plasmids are first bound to surfaces and then interrogated with a specific target drug, which is conjugated to a fluorescent moiety. The incorporated affinity tags are used for the binding of the *in vitro* expressed protein to a specially prepared surface containing a capture molecule which interacts with the affinity tag. Incorporated fluorophores are used to monitor surface binding. Target drug binding is detected using dual wavelength fluorescence detection or alternatively fluorescence resonance energy transfer, which assures that the protein and bound drug are in close proximity. The drug-protein complex can also be released using a photocleavable biotin linker, for downstream analysis to confirm the drug-protein interaction and for further mass spectrometric analysis of the drug or protein identity.

In both scenarios described above, parallel expression of the genomic library, which is estimated to consist of 2000 individual plasmid pools of approximately 50 plasmids per pool, provides the basis to perform rapid drug-proteomic analysis. For example, using a preformatted set of reaction wells (50 microliters each) and plasmid sets, the entire parallel expression reaction could be accomplished in less than 1 hour. The purification of the expressed proteins from each sample could also be extremely rapid, provided that suitable equipment (e.g. 96 well-pipetters operated by robotic arms) were utilized. For example, pipette filter tips can be prepared that contain an affinity media for capture via the *in vitro* incorporated photocleavable tag (e.g. streptavidin bound to beads) and light can be used for subsequent release.

In both scenarios, the samples can be rapidly transferred to a surface for

subsequent drug-protein fluorescent assays. For example, in the case of slides coated with the target drug, individual samples can be spotted using a commercial arrayer such as the Affymetrix 417 arrayer, which offers 125 um resolution spot size. In principle, a single microscope slide would be sufficient to array the entire proteome with 5-fold redundancy in less than 10 minutes. Larger spots sizes or greater redundancy would require more slides, however the entire preparation of the arrayed proteome would still rapid and amenable to high throughput processing. The entire "proteome on a slide" concept is particularly attractive, since the rapid production of such slides would enable screening of the entire proteome versus libraries of drugs.

For the above mentioned format, rapid read-out can be achieved using a fluorescent 2-D microarray reader. For example, the Affymetrix 418 scanner can scan an entire slide in approximately 10 minutes and is able to perform 3 color imaging. Furthermore, the array reader offers high precision (10 microns), speed (18 mm/min ) and sensitivity (less than 1 Cy3 dye molecules per 1 square micron with > 3.5 S/N).

In order to evaluate the screening assays of the present invention, experiments were performed which involved the incorporation of PC-Biotin or BODIPY into the test protein dihydrofolate reductase (DHFR). Experiments were aimed at evaluating whether PC-Biotin or BODIPY markers incorporated into DHFR during its *in vitro* synthesis could be used to assay for its interaction with the small ligand methotrexate, a competitive inhibitor for DHFR whose normal ligand is dihydrofolate. Since methotrexate can reduce the rate of cell growth, it is widely used as an anticancer drug in chemo-therapy .

In one assay, PC-Biotin was incorporated into DHFR during its *in vitro* expression in S30 in the presence of PC-Biotin-Lys-tRNA. After expression, the crude mixture was exposed to methotrexate-coated agarose beads. After extensive washing, specific binding of the DHFR to the beads was assayed using streptavidin-HRP. As seen in Figure 40, a signal is obtained only when PC-Biotin-Lys-tRNA is included in the expression system. UV-light exposure prior to binding, abolishes the signal relative to control levels (data not shown), providing a convenient method to measure background levels during automated assays.

In a second experiment, capillary electrophoresis (CE-LIF) was used as a homogenous solution assay to detect the interaction of DHFR and methotrexate. In this case, BODIPY was incorporated at the N-terminus of DHFR during its *in vitro* synthesis using BODIPY-fmet-tRNA. The electropherogram for free DHFR exhibited a peak at 2.9 minutes (data not shown). In comparison, the electropherogram of DHFR preincubated with methotrexate for 30 minutes at room temperature exhibited a broadened peak at 2.8 min (data not shown). The change in migration time is attributed to the complex formation between DHFR and methotrexate.

In a third series of experiments, the ability to selectively bind and detect an *in vitro* expressed target protein using an antibody was explored. For this purpose, PC-Biotin was incorporated into p53 expressed in rabbit reticulocyte lysate after RT-PCR of a pooled human mRNA sample using the PC-Biotin-Lys-tRNA. The protein was then specifically captured on ELISA plates using monoclonal antibody clone BP53-12 (Santa Cruz Biotechnology, Santa Cruz, CA) which recognizes both WT and mutant conformations. As a negative control, an identical quantity of pre-immune mouse IgG was used for capture, in place of the p53 specific antibody. Detection of the biotin was achieved using peroxidase labeled NeutrAvidin biotin binding protein (Pierce, Rockford, IL) in a standard ELISA assay. The results (not shown) indicated that only specific binding with the p53 antibody results in a significant signal. These data demonstrate the feasibility of studying p53 binding using a panel of conformation specific antibodies which selectively react with different p53 mutants.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered exemplary only, with the scope of particular embodiments of the invention indicated by the following claims.

## CLAIMS

1. A method, comprising:
  - a) providing a tRNA molecule and a BODIPY marker; and
  - b) aminoacylating said tRNA molecule with said BODIPY marker to create  
5 a misaminoacylated tRNA.
2. The method of Claim 1, further comprising:
  - c) introducing said misaminoacylated tRNA into a translation system under conditions such that said marker is incorporated into a nascent protein.
3. The method of Claim 2, further comprising:
  - d) detecting said nascent protein containing said marker.  
10
4. The method of Claim 3, further comprising:
  - e) isolating said detected nascent protein.
5. The method of Claim 3, wherein the nascent protein detected is selected from recombinant gene products, gene fusion products, enzymes, cytokines, carbohydrate  
15 and lipid binding proteins, nucleic acid binding proteins, hormones, immunogenic proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.
6. The method of Claim 2, wherein the translation system comprises a cellular or cell-free translation system.
7. The method of Claim 5, wherein the cellular translation system is selected from  
20 the group consisting of tissue culture cells, primary cells, cells *in vivo*, isolated immortalized cells, human cells and combinations thereof.

8. The method of Claim 5, wherein the cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof.
9. The method of Claim 5, wherein said cell-free translation system is incubated at a temperature of between about 25°C to about 45°C.
10. The method of Claim 5, wherein the cell-free translation system is a continuous flow or dialysis system.
11. The method of Claim 1, wherein the tRNA molecule is aminoacylated by chemical or enzymatic misaminoacylation.
12. The method of Claim 2, wherein two or more different misaminoacylated tRNAs are introduced into the translation system.
13. The method of Claim 3, wherein said nascent protein detected is functionally active.
14. The method of Claim 1, wherein said tRNA molecule is an initiator tRNA molecule.
15. The method of Claim 1, wherein said tRNA molecule is a suppressor tRNA molecule.



16. A kit, comprising:

- a) a first containing means containing at least one component of a protein synthesis system;
- b) a second containing means containing a misaminoacylated tRNA, wherein said tRNA is misaminoacylated with a BODIPY marker.

17. The kit of Claim 16, wherein said misaminoacylated tRNA comprises an initiator tRNA.

18. The kit of Claim 16, wherein said misaminoacylated tRNA comprises a suppressor tRNA.

19. The kit of Claim 16, wherein said component of said protein synthesis system comprises ribosomes.

20. A tRNA molecule misaminoacylated with a BODIPY marker.

21. A method, comprising:

- a) providing: i) a misaminoacylated initiator tRNA molecule which only recognizes the first AUG codon that serves to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising an affinity marker and a C-terminal marker; and
- b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker, said affinity marker and said C-terminal marker.

22. The method of Claim 21, wherein the nascent protein generated is selected from recombinant gene products, gene fusion products, enzymes, cytokines, carbohydrate and lipid binding proteins, nucleic acid binding proteins, hormones, immunogenic proteins, truncated proteins, mutant proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.
23. The method of Claim 21, wherein the translation system comprises a cellular or cell-free translation system.
24. The method of Claim 23, wherein the cellular translation system is selected from prokaryotic cells, eukaryotic cells, tissue culture cells, primary cells, cells *in vivo*, isolated immortalized cells, human cells and combinations thereof.
25. The method of Claim 23, wherein the cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof.
26. The method of Claim 23, wherein said cell-free translation system is incubated at a temperature of between about 25°C to about 45°C.
27. The method of Claim 23, wherein the cell-free translation system is a continuous flow or dialysis system.
28. The method of Claim 21, wherein the tRNA molecule is aminoacylated by chemical or enzymatic misaminoacylation.
29. The method of Claim 23, wherein said nascent protein is functionally active.

30. The method of Claim 21, wherein said first marker comprises a fluorescent compound.

31. The method of Claim 30, wherein said fluorescent compound is selected from dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes and derivatized coumarin.

32. The method of Claim 21, wherein said C-terminal comprises a histidine tag.

33. A method, comprising:

a) providing i) a misaminoacylated initiator tRNA molecule which only recognizes the first AUG codon that serves to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising an affinity marker and a C-terminal marker; and

b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker at the N-terminus of said protein, a C-terminal marker, and said affinity marker adjacent to said first marker.

34. The method of Claim 33, wherein the nascent protein generated is selected from recombinant gene products, gene fusion products, enzymes, cytokines, carbohydrate and lipid binding proteins, nucleic acid binding proteins, hormones, immunogenic proteins, truncated proteins, mutant proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.

35. The method of Claim 33, wherein the translation system comprises a cellular or cell-free translation system.

36. The method of Claim 35, wherein the cellular translation system is selected

from prokaryotic cells, eukaryotic cells, tissue culture cells, primary cells, cells *in vivo*, isolated immortalized cells, human cells and combinations thereof.

37. The method of Claim 35, wherein the cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof.
38. The method of Claim 35, wherein said cell-free translation system is incubated at a temperature of between about 25°C to about 45°C.
39. The method of Claim 35, wherein the cell-free translation system is a continuous flow or dialysis system.
40. The method of Claim 33, wherein the tRNA molecule is aminoacylated by chemical or enzymatic misaminoacylation.
41. The method of Claim 33, wherein said nascent protein is functionally active.
42. The method of Claim 33, wherein said first marker comprises a fluorescent compound.
43. The method of Claim 42, wherein said fluorescent compound is selected from dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes and derivatized coumarin.
44. The method of Claim 33, wherein said C-terminal comprises a histidine tag.

45. A method, comprising:
- a) providing i) a misaminoacylated tRNA molecule which only recognizes the first codon designed to serve to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising an affinity marker and a C-terminal marker; and
  - b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker, said affinity marker and said C-terminal marker.
46. The method of Claim 45, wherein the nascent protein generated is selected from recombinant gene products, gene fusion products, enzymes, cytokines, carbohydrate and lipid binding proteins, nucleic acid binding proteins, hormones, immunogenic proteins, truncated proteins, mutant proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.
47. The method of Claim 45, wherein the translation system comprises a cellular or cell-free translation system.
48. The method of Claim 47, wherein the cellular translation system is selected from prokaryotic cells, eukaryotic cells, tissue culture cells, primary cells, cells *in vivo*, isolated immortalized cells, human cells and combinations thereof.

49. The method of Claim 47, wherein the cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof.
50. The method of Claim 47, wherein said cell-free translation system is incubated at a temperature of between about 25°C to about 45°C.
51. The method of Claim 47, wherein the cell-free translation system is a continuous flow or dialysis system.
52. The method of Claim 45, wherein the tRNA molecule is aminoacylated by chemical or enzymatic misaminoacylation.
53. The method of Claim 45, wherein said nascent protein is functionally active.
54. The method of Claim 45, wherein said first marker comprises a fluorescent compound.
55. The method of Claim 44, wherein said fluorescent compound is selected from dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes and derivatized coumarin.
56. The method of Claim 45, wherein said C-terminal comprises a histidine tag.

57. A method, comprising:

a) providing i) a misaminoacylated tRNA molecule which only recognizes the first codon designed to serve to initiate protein synthesis, said misaminoacylated initiator tRNA molecule comprising a first marker, and ii) a nucleic acid template encoding a protein, said protein comprising an affinity marker and a C-terminal marker; and

b) introducing said misaminoacylated initiator tRNA to a translation system comprising said template under conditions such that a nascent protein is generated, said protein comprising said first marker at the N-terminus of said protein, a C-terminal marker, and said affinity marker adjacent to said first marker.

58. The method of Claim 57, wherein the nascent protein generated is selected from recombinant gene products, gene fusion products, enzymes, cytokines, carbohydrate and lipid binding proteins, nucleic acid binding proteins, hormones, immunogenic proteins, truncated proteins, mutant proteins, human proteins, viral proteins, bacterial proteins, parasitic proteins and fragments and combinations thereof.

59. The method of Claim 58, wherein the translation system comprises a cellular or cell-free translation system.

60. The method of Claim 59, wherein the cellular translation system is selected from prokaryotic cells, eukaryotic cells, tissue culture cells, primary cells, cells *in vivo*, isolated immortalized cells, human cells and combinations thereof.

61. The method of Claim 59, wherein the cell-free translation system is selected from the group consisting of *Escherichia coli* lysates, wheat germ extracts, insect cell lysates, rabbit reticulocyte lysates, frog oocyte lysates, dog pancreatic lysates, human cell lysates, mixtures of purified or semi-purified translation factors and combinations thereof.

62. The method of Claim 59, wherein said cell-free translation system is incubated at a temperature of between about 25°C to about 45°C.

63. The method of Claim 59, wherein the cell-free translation system is a continuous flow or dialysis system.

5 64. The method of Claim 57, wherein the tRNA molecule is aminoacylated by chemical or enzymatic misaminoacylation.

65. The method of Claim 57, wherein said nascent protein is functionally active.

66. The method of Claim 57, wherein said first marker comprises a fluorescent compound.

10 67. The method of Claim 66, wherein said fluorescent compound is selected from dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes and derivatized coumarin.

68. The method of Claim 57, wherein said C-terminal comprises a histidine tag.



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(54) Title: METHODS FOR THE DETECTION, ANALYSIS AND ISOLATION OF NASCENT PROTEINS

(57) Abstract: This invention relates to non-radioactive markers that facilitate the detection and analysis of nascent proteins translated within cellular or cell-free translation systems. Nascent proteins containing these markers can be rapidly and efficiently detected, isolated and analyzed without the handling and disposal problems associated with radioactive reagents. Preferred markers are dipyrrometheneboron difluoride (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes.

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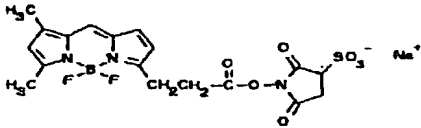
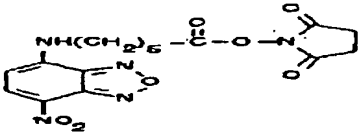
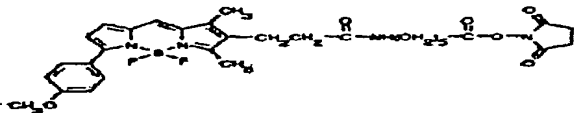
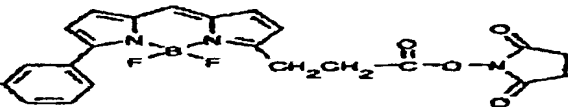
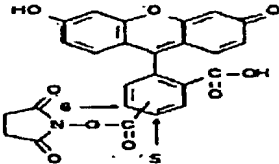
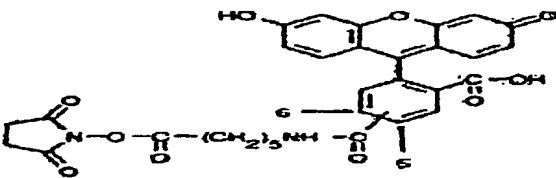
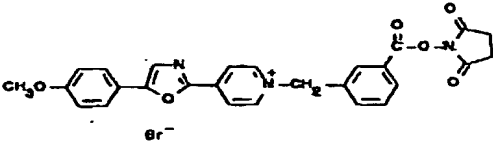
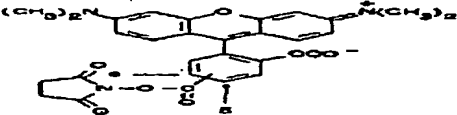
Name and Molecular weight	Formula	Fluorescence Properties
BODIPY-FL, SSE M. WT. 491		Excitation=502 nm Emmision=510 nm Extinction=75,000
NBD M. WT. 391		Excitation=466 nm Emmision=535 nm Extinction=22,000
Bodipy-TMR-X, SE M. WT. 608		Excitation=544 nm Emmision=570 nm Extinction=56,000
Bodipy-R6G M. WT. 437		Excitation=528 nm Emmision=547 nm Extinction=70,000
Fluorescein (FAM) M. WT. 473		Excitation=495 nm Emmision=520 nm Extinction=74,000
Fluorescein (SFX) M. WT. 587		Excitation=494 nm Emmision=520 nm Extinction=73,000
PyMPO M. WT. 582		Excitation=415 nm Emmision=570 nm Extinction=26,000
5/6-TAMRA M. WT. 528		Excitation=546 nm Emmision=576 nm Extinction=95,000

Table 2

Enzyme/Protein	-FluoroTag™ tRNA Translation reaction	+FluoroTag™ tRNA Translation reaction
$\alpha$ -Hemolysin OD <sub>415nm</sub> /μl	0.085	0.083
Luciferase RLU/μl	79052	78842
DHFR $\Delta$ OD <sub>339nm</sub> /μl	0.050	0.064

**Table 3**

## Various Epitopes and Their Sequences

Name	Amino acid sequence	Nucleotide sequence (5'→ 3')
His-6	HHHHHH	CATCACCATCACCATCAC
FLAG	DYKDDDDK	GACTACAAGGACGACGACGACAAG
c-Myc	EQKLISEEDL	GAGCAGAAGCTGATCAGCGAGGAGGACCTG
Strep-Tag	WSHPQFEK	TGGAGCCACCCCCAGTTCGAGAAG
Amber-16	CSPFEVQVSPEA GAQK	TGCAGCCCCTTCGAGGTGCAGGTGAGCCCCGAGGCCGGCGCC CAGAAG
T7-Tag	MASMTGGQQMG	ATGGCCAGCATGACCGGCGGCCAGCAGATGGGC
VSV-G <sup>#</sup> -Tag	YTDIEMNRLGK	TACACCGACATCGAGATGAACCGCCTGGGCAAG
HA*-Tag	YPYDVPDYA	TACCCCTACGACGTGCCCGACTACGCC
Protein-C Tag	EDQVDPRLIDGK	GAGGACCAGGTGGACCCCCGCCTGATCGACGGCAAG
HSV <sup>\$</sup> -Tag	QPELAPEDPED	CAGCCCGAGCTGGCCCCCGAGGACCCCGAGGAC

# - Vesicular Stomatitis Virus Glycoprotein

\* - HemAgglutinin

\$ - Herpes Simplex Virus glycoprotein

## TABLE 4

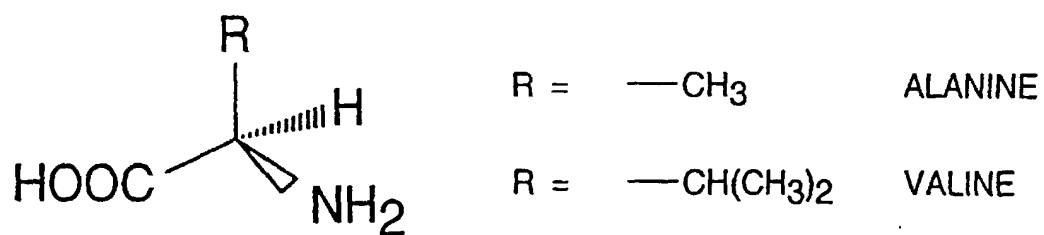
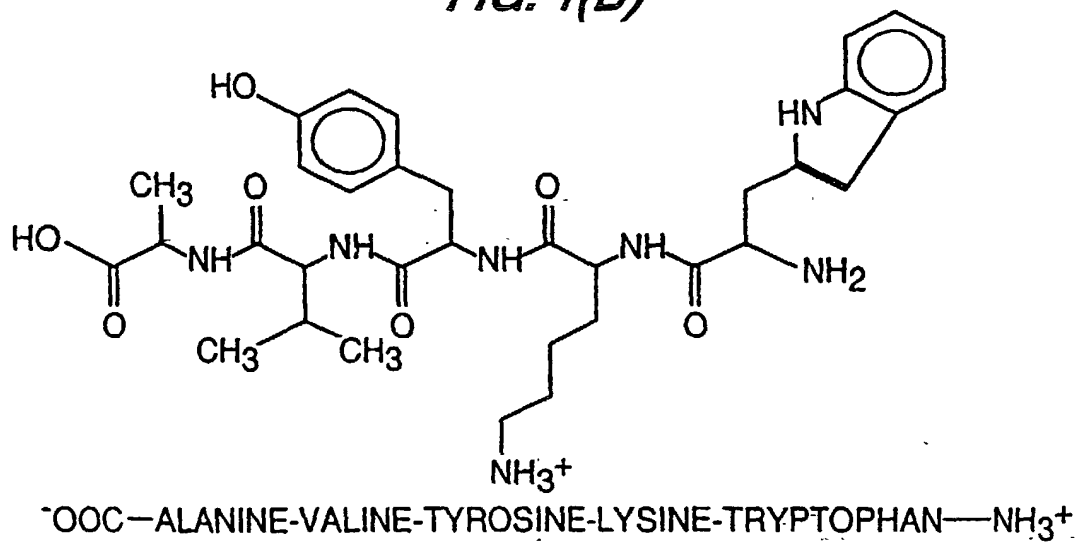
**FIG. 1(A)****FIG. 1(B)**

FIG. 2

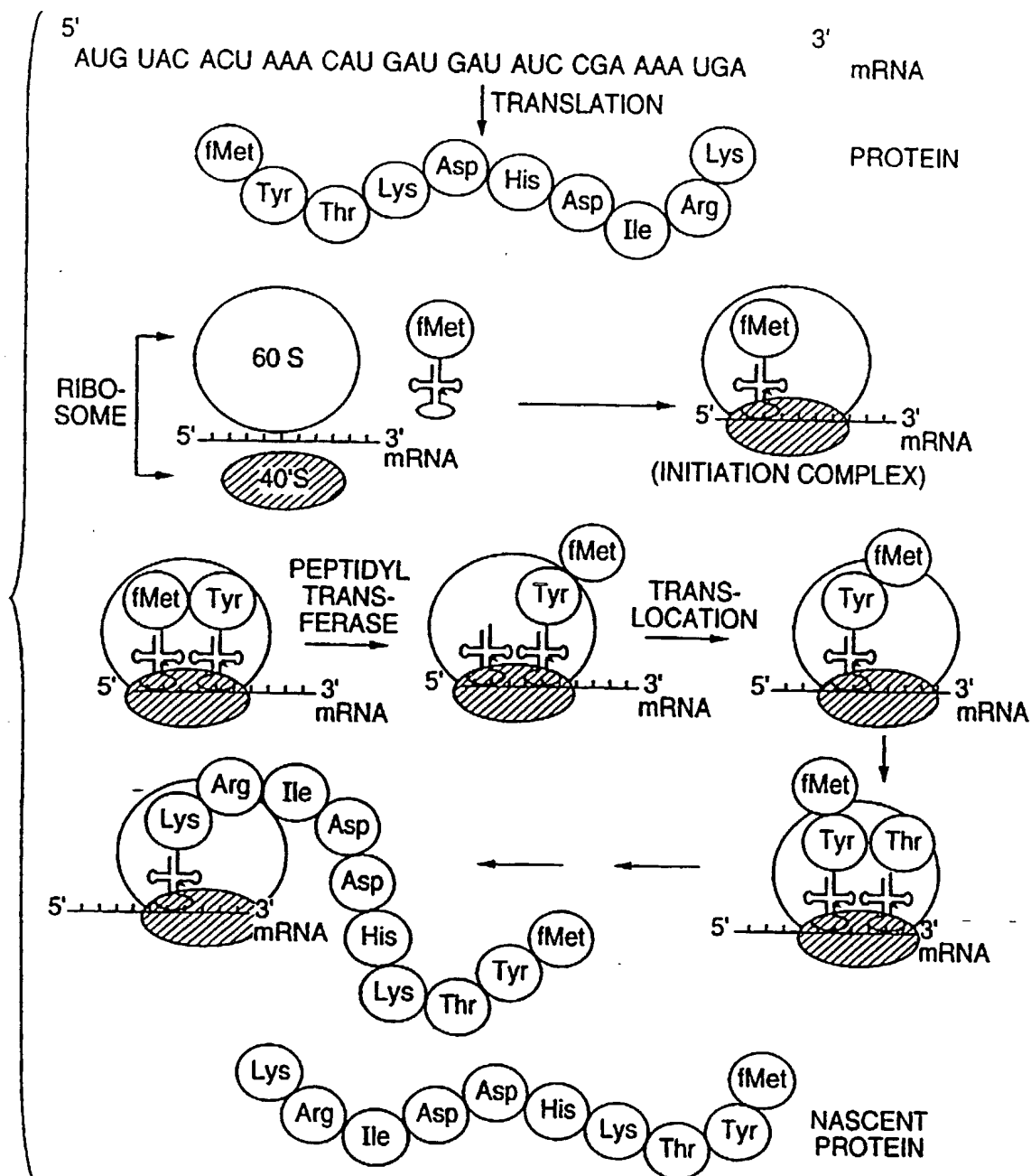


FIG. 3(A)

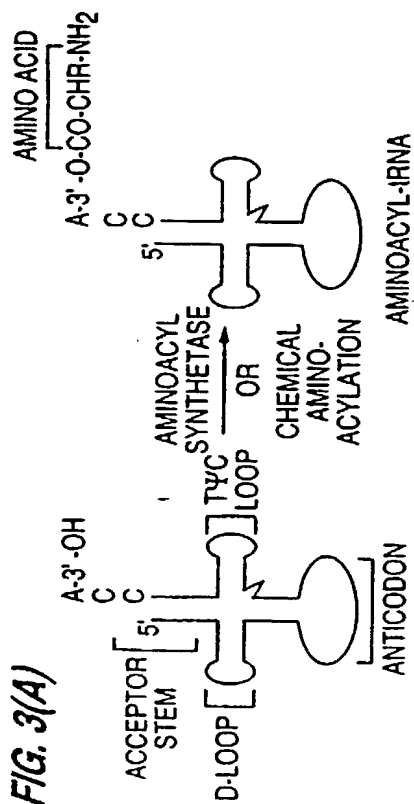
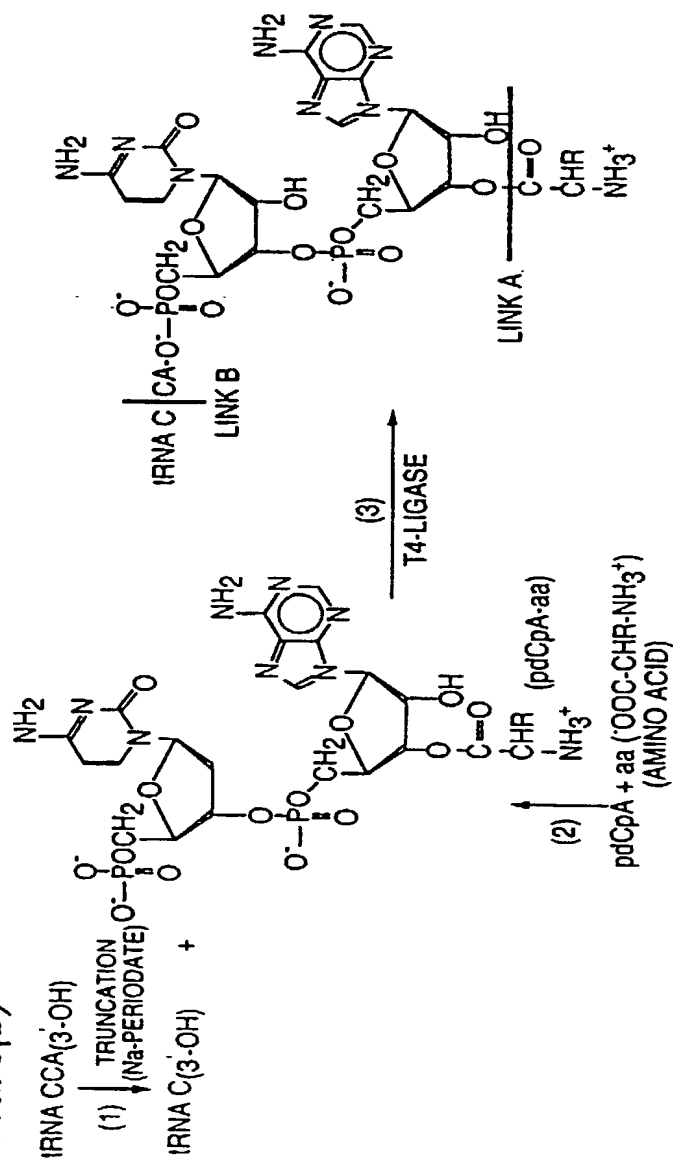
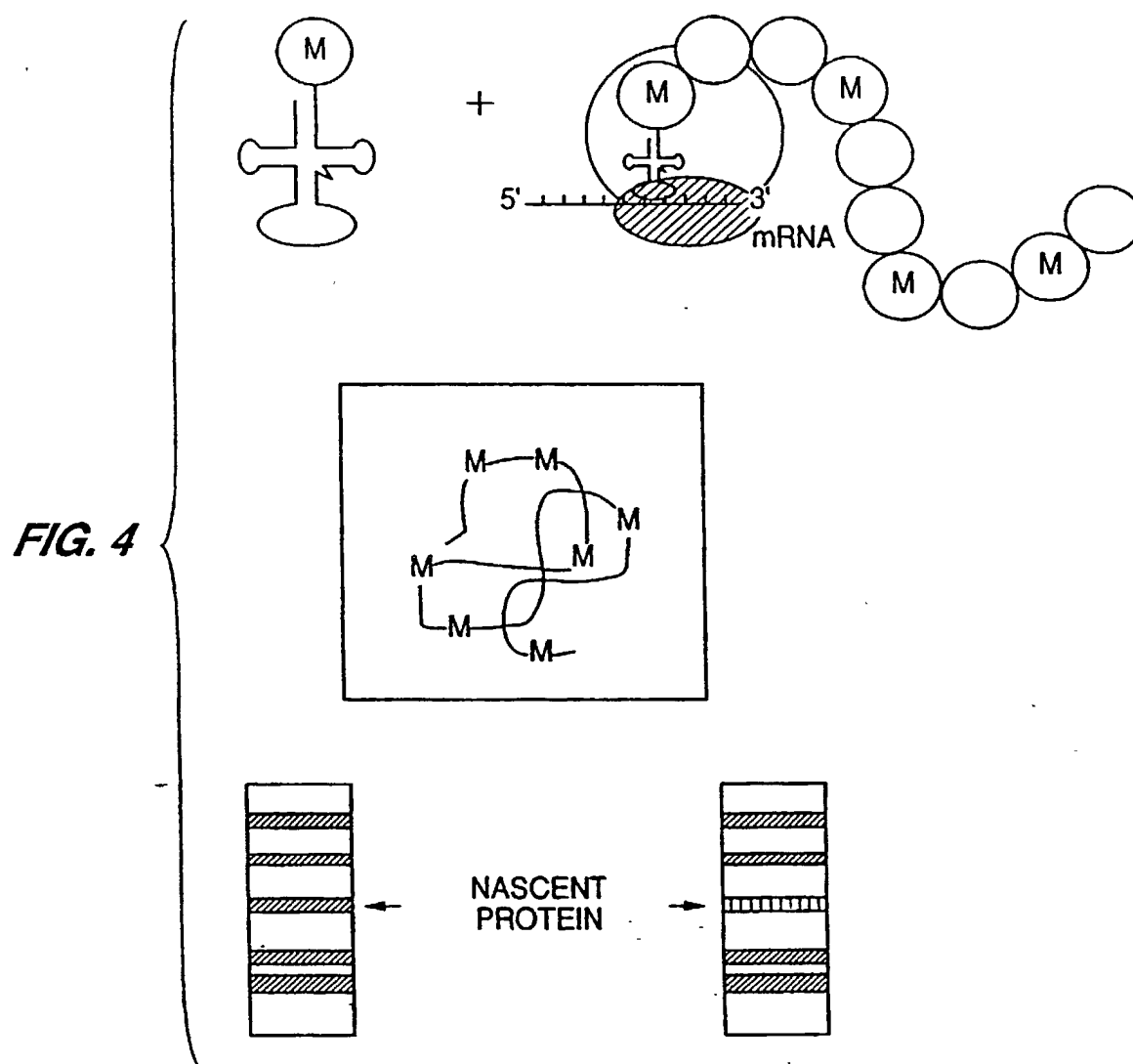


FIG. 3(B)







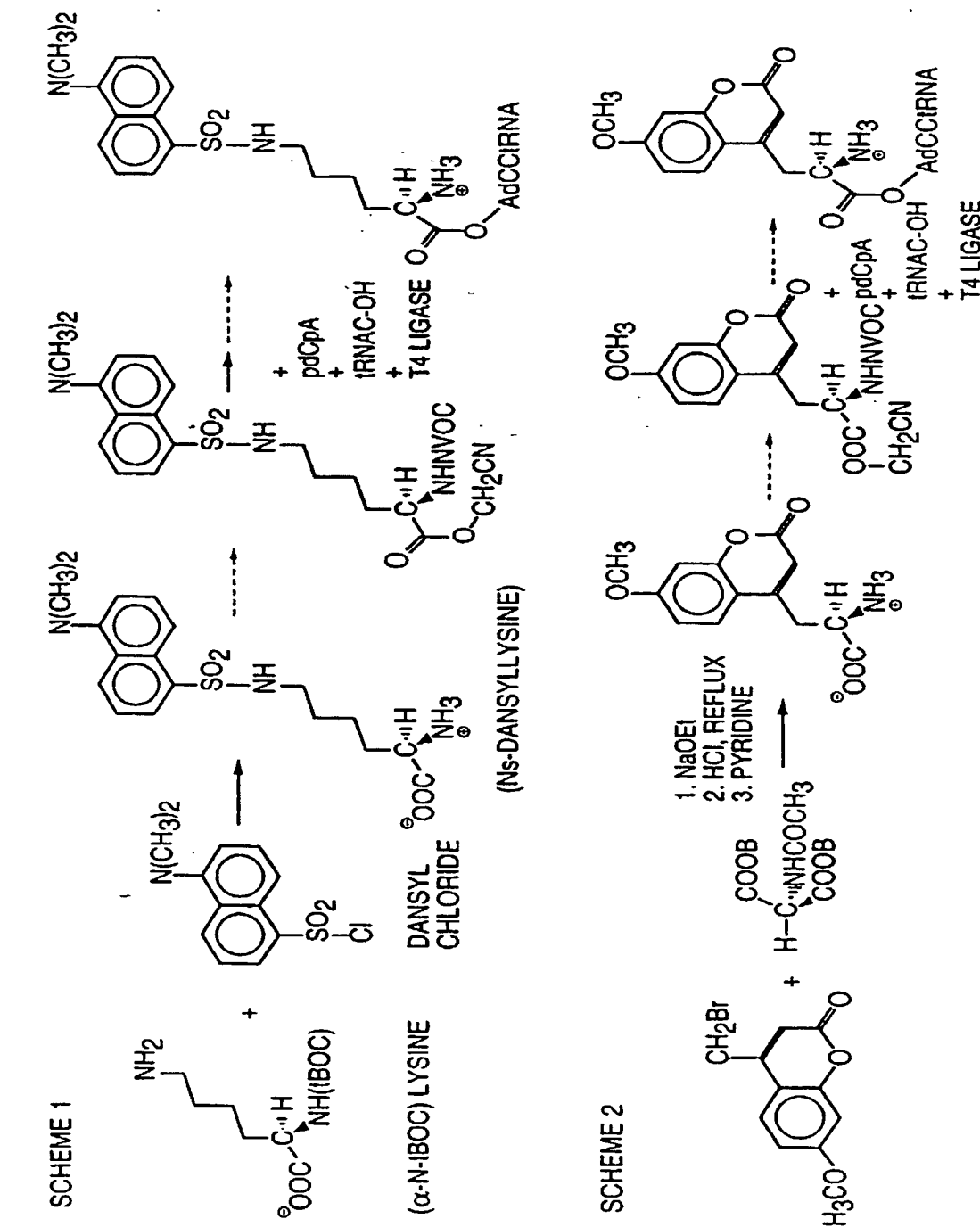


FIG. 5

FIG. 6(A)

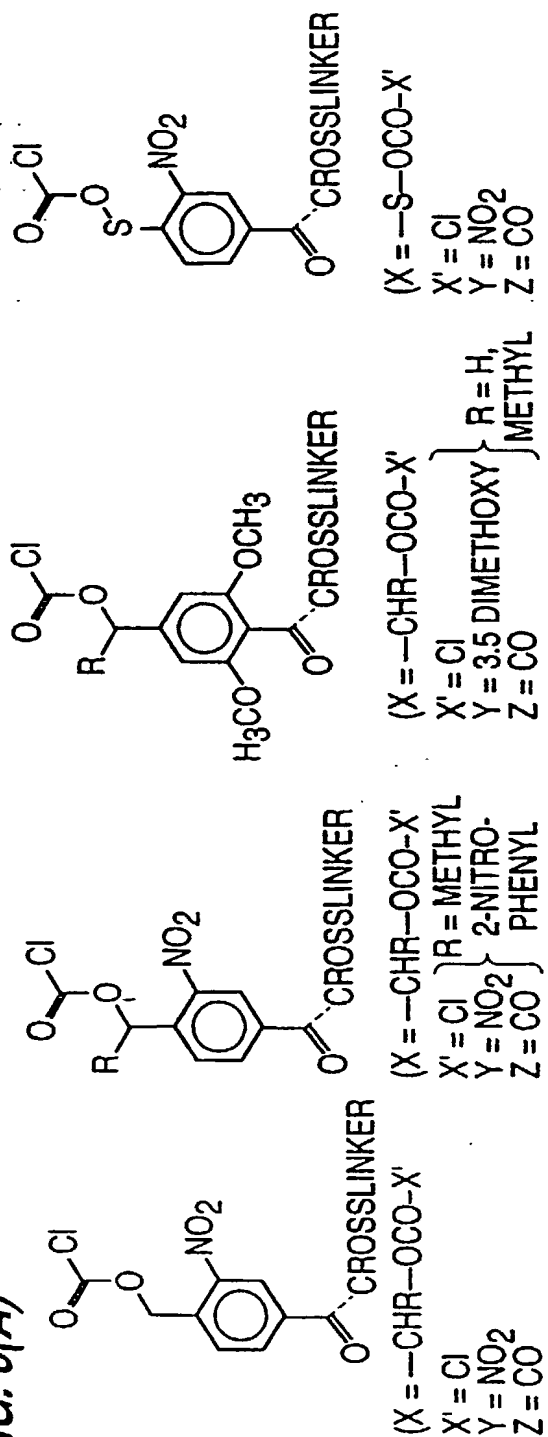
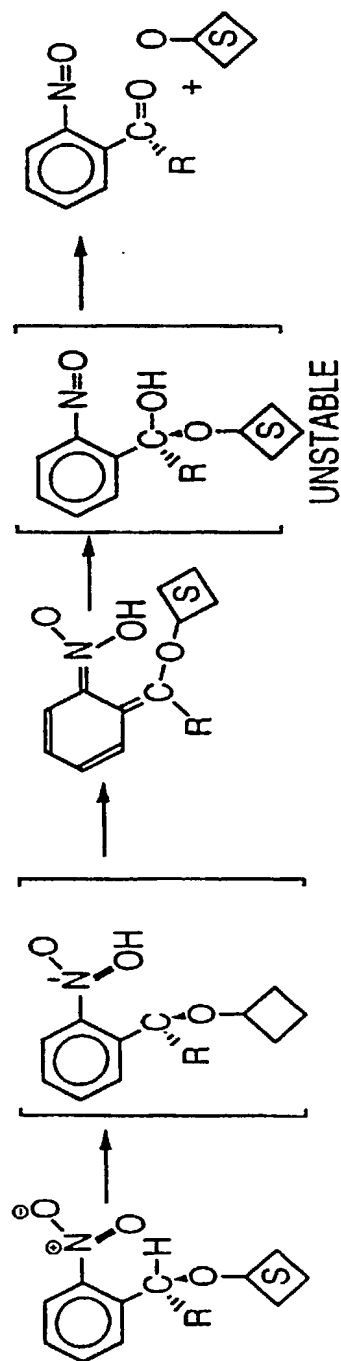


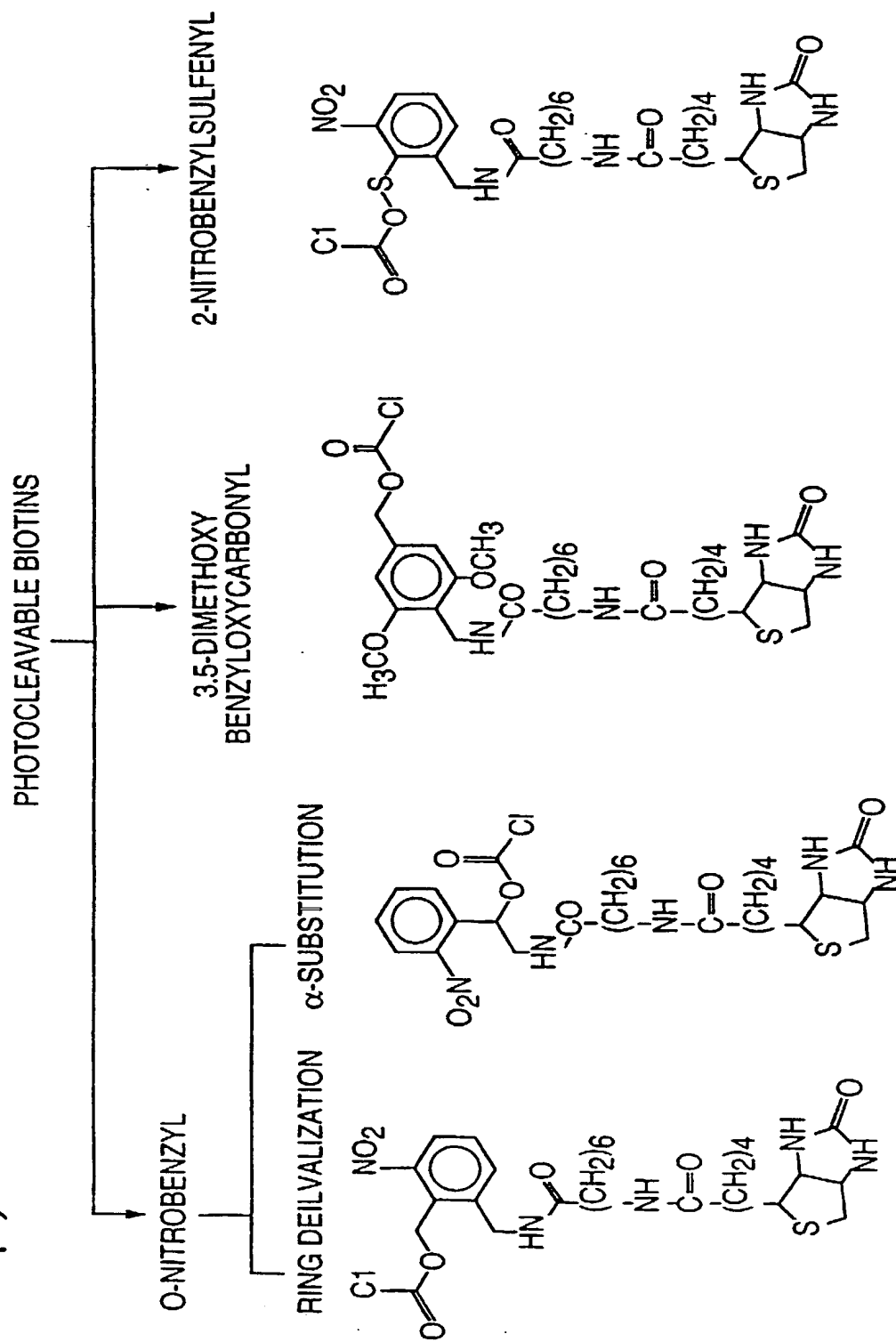
FIG. 6(B)



**10/53**

10/03/93

FIG. 8(A)



10/049332

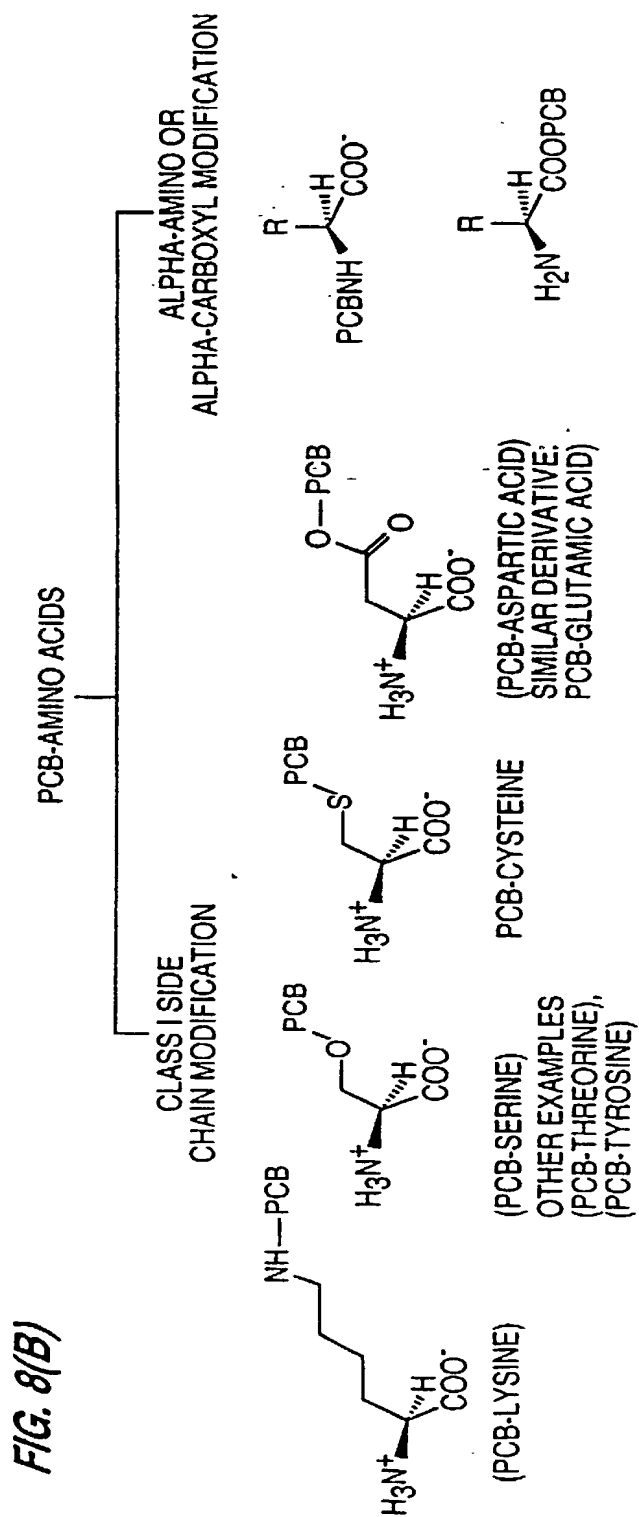
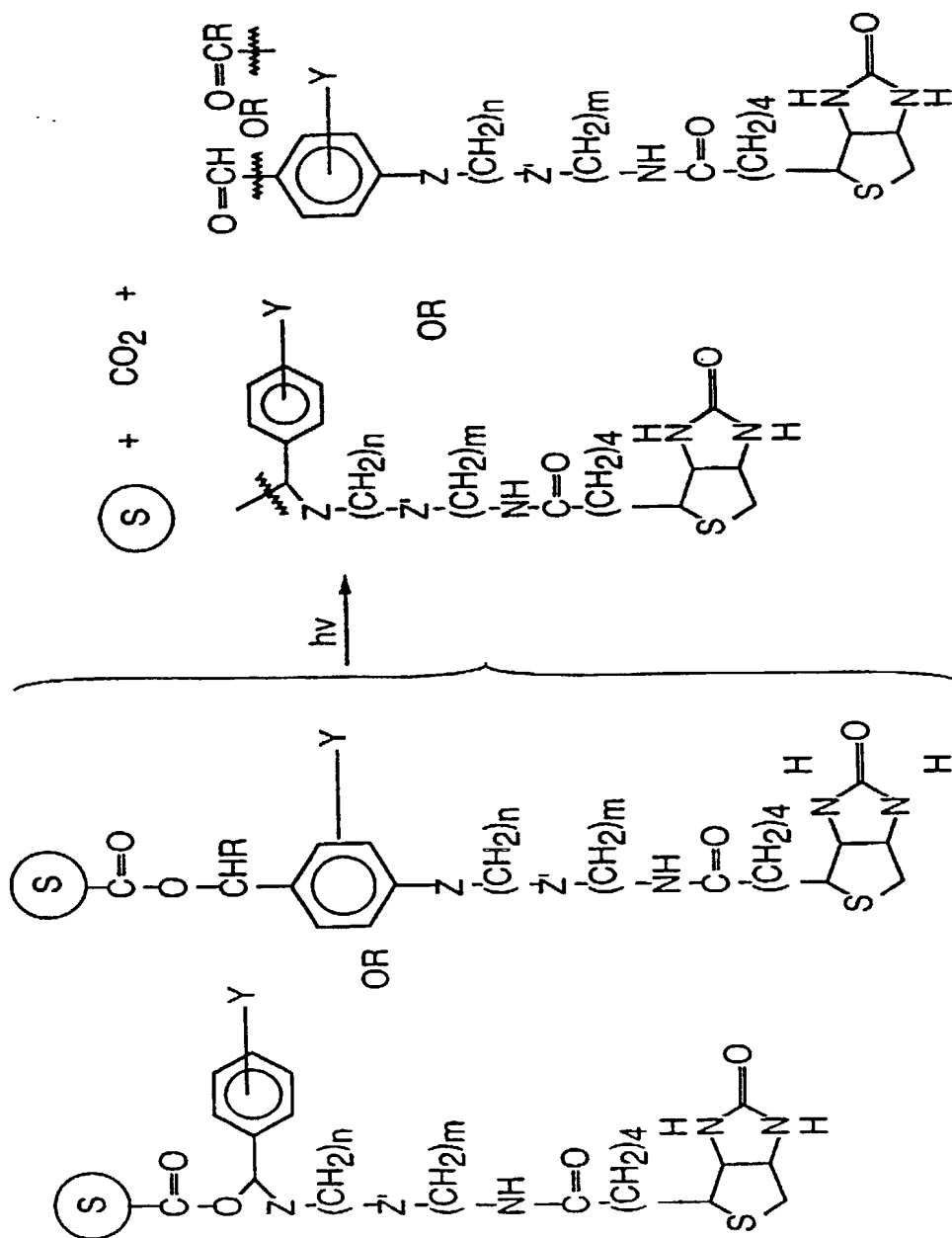
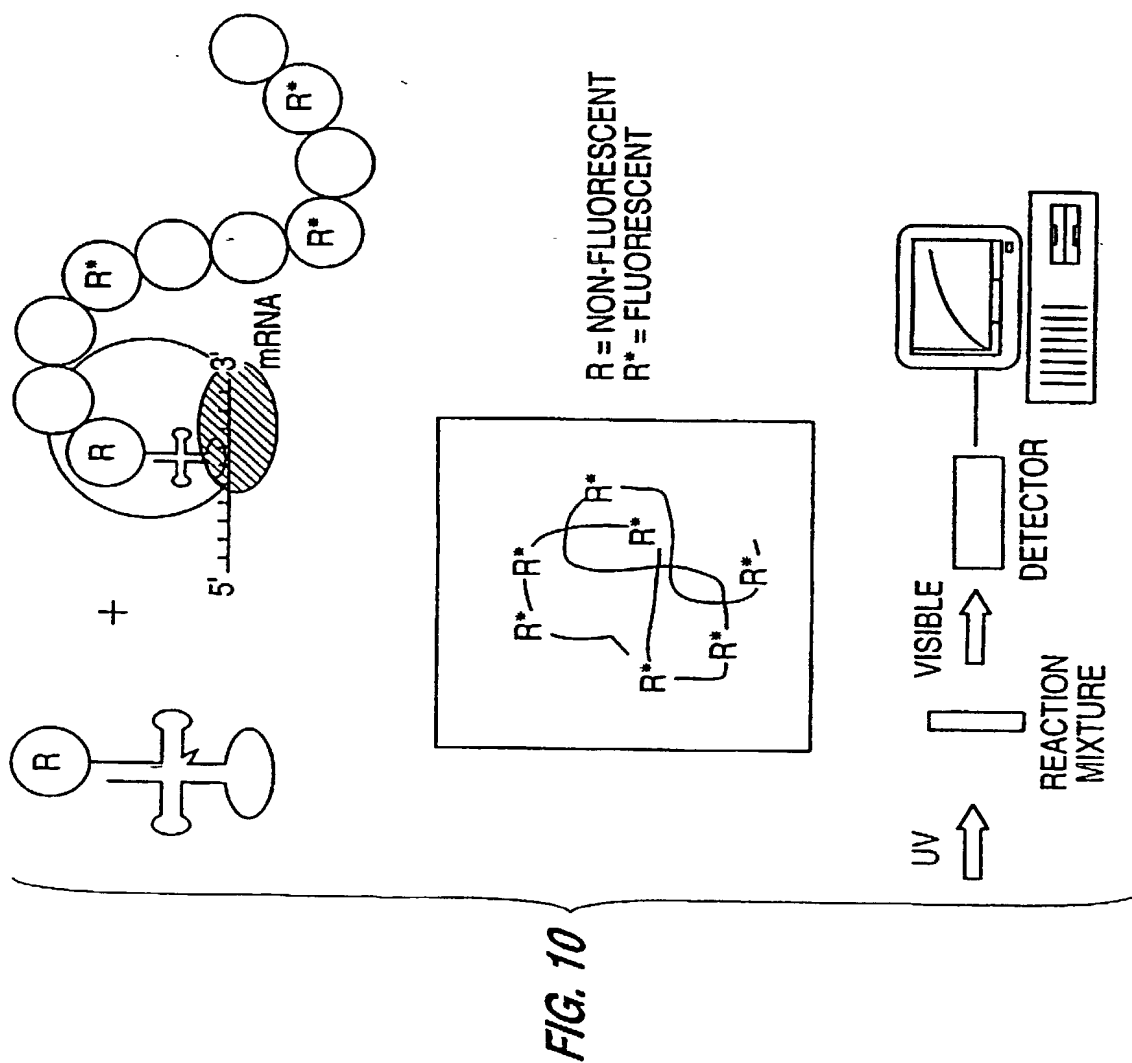


FIG. 9





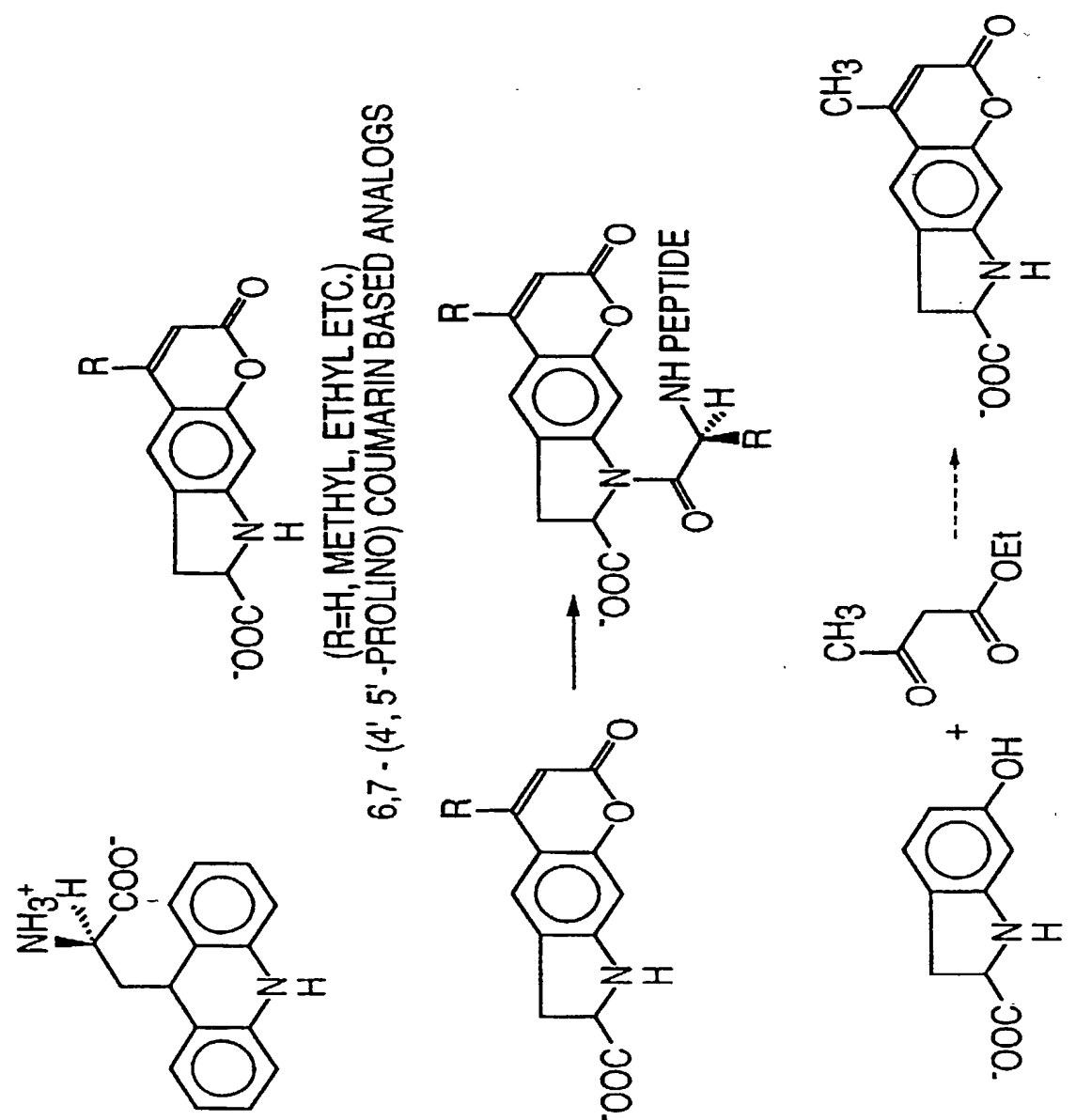


FIG. 11



**FIG. 12**

$$\begin{array}{c} \text{X}' \\ | \\ \text{C=O} \\ | \\ \text{O} \\ | \\ \text{CHR} \\ | \\ \text{OR} \end{array}$$

$$\text{X}' = \text{Cl, O-NHYDROXSUCCINIMIDYL, O-CH}_2\text{CN, OPbF}_5, \text{OPhCl}_5, \text{N}_3 \text{ ETC. (REACTIVE DERIVATIVE)}$$

$$\text{R} = \text{H, ALKYL, SUBSTITUTED ALKYL, ARYL, SUBSTITUTE ARYL}$$

$$\text{X} = \left\{ \begin{array}{l} \text{X}' - \text{C=O} - \text{O} - \text{CH}_2 - \text{C}_6\text{H}_4 - \text{Y} \\ \text{Z} - (\text{CH}_2)_n - \text{Z}' - (\text{CH}_2)_m - \text{NH} - \text{C=O} - (\text{CH}_2)_4 - \text{BLOTNYL MOIETY} \end{array} \right\}$$

$$\text{Y} = (\text{POLY}) \text{NO}_2, \text{ALKYL, ALKOXYL}$$

$$\text{Z} = \text{C} \begin{array}{c} \text{O} \\ || \\ \text{O} \end{array} \text{C} \begin{array}{c} \text{O} \\ || \\ \text{O} \end{array} \text{N} - \text{C} \begin{array}{c} \text{O} \\ || \\ \text{O} \end{array} \text{NH, O, S, CH}_2$$

$$n = 0, 1, 2, \dots (n = 0, Z = 0)$$

$$Z' = \text{AS FOR Z (Z = Z' OR Z} \neq \text{Z')}$$

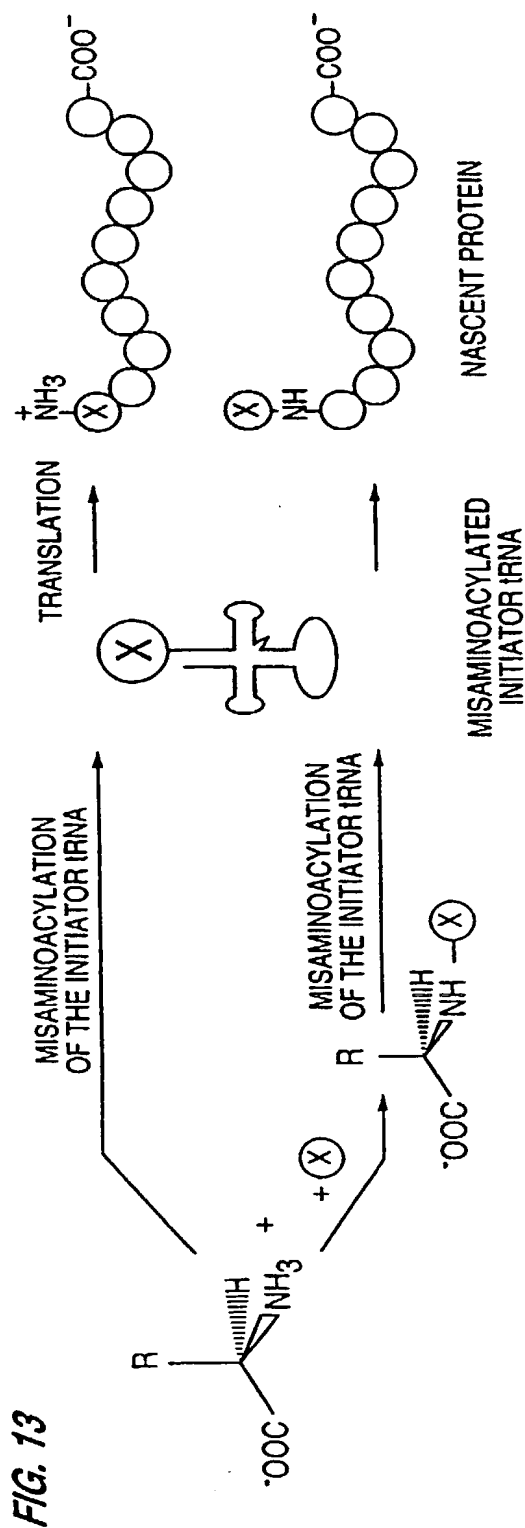
$$m = 0, 1, 2, \dots (m = 0, Z = 0)$$

**PCB**

**PHOTO-REACTIVE MOIETY**

**LINEAR ARM**

**BLOTNYL MOIETY**



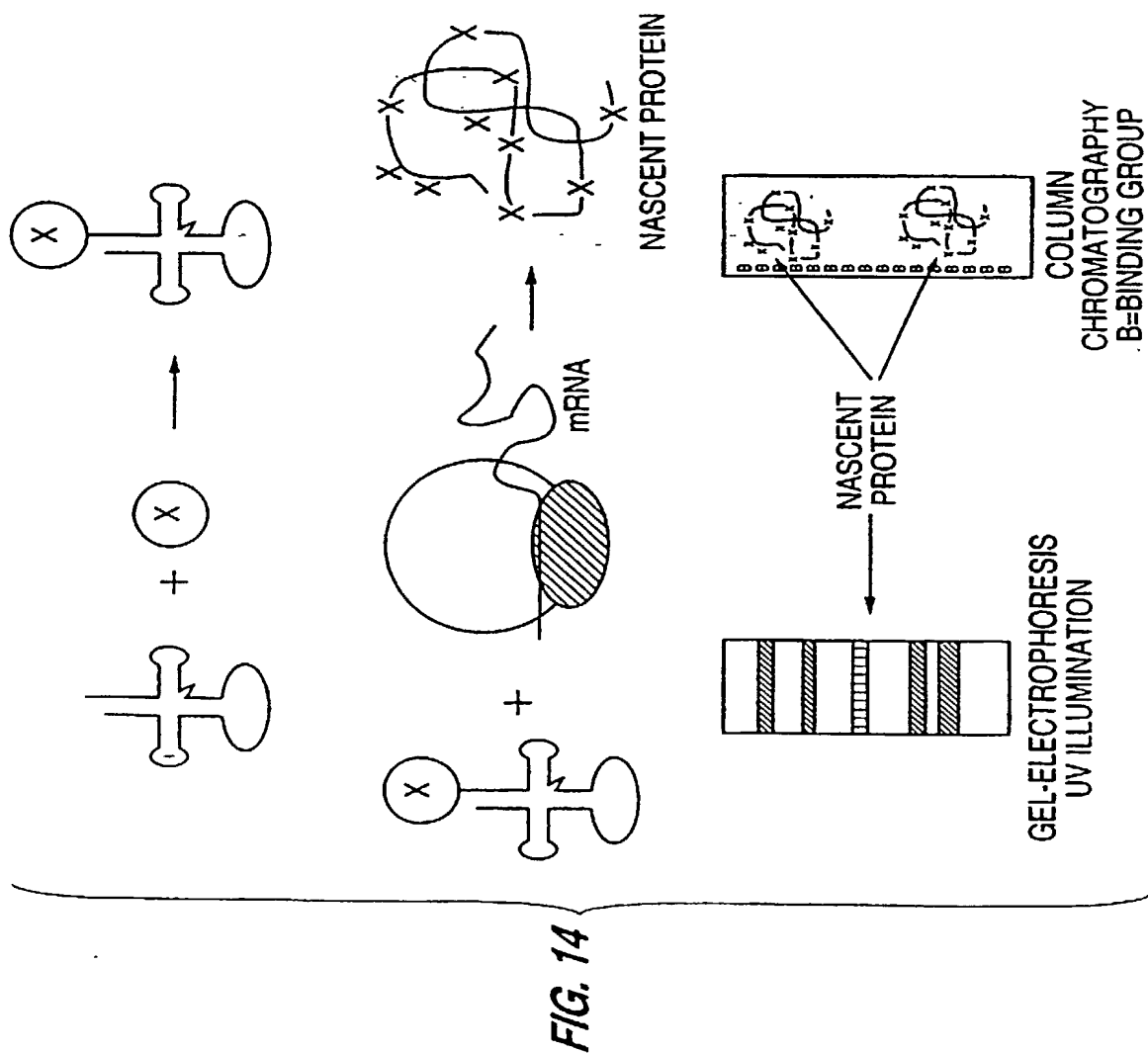
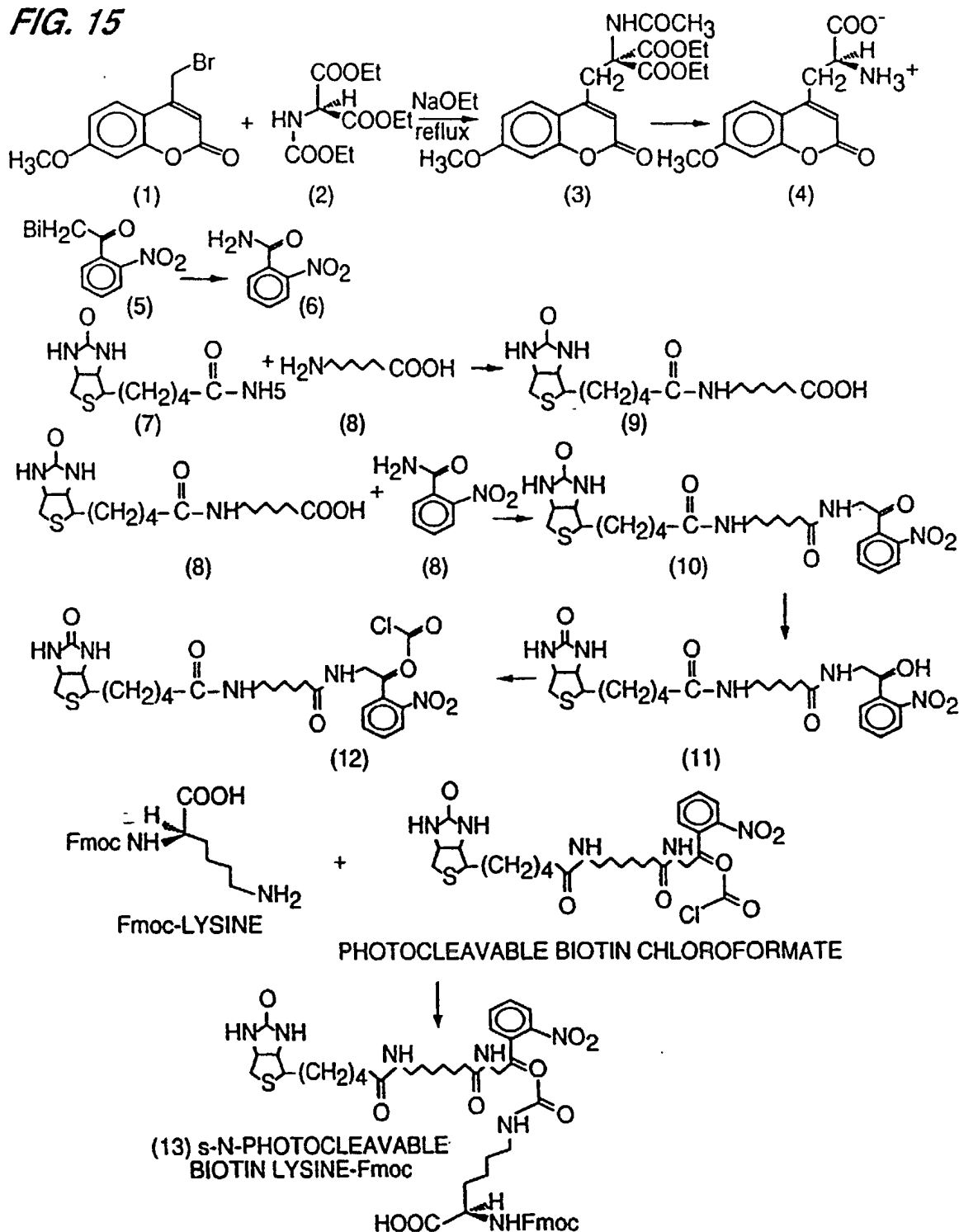


FIG. 15



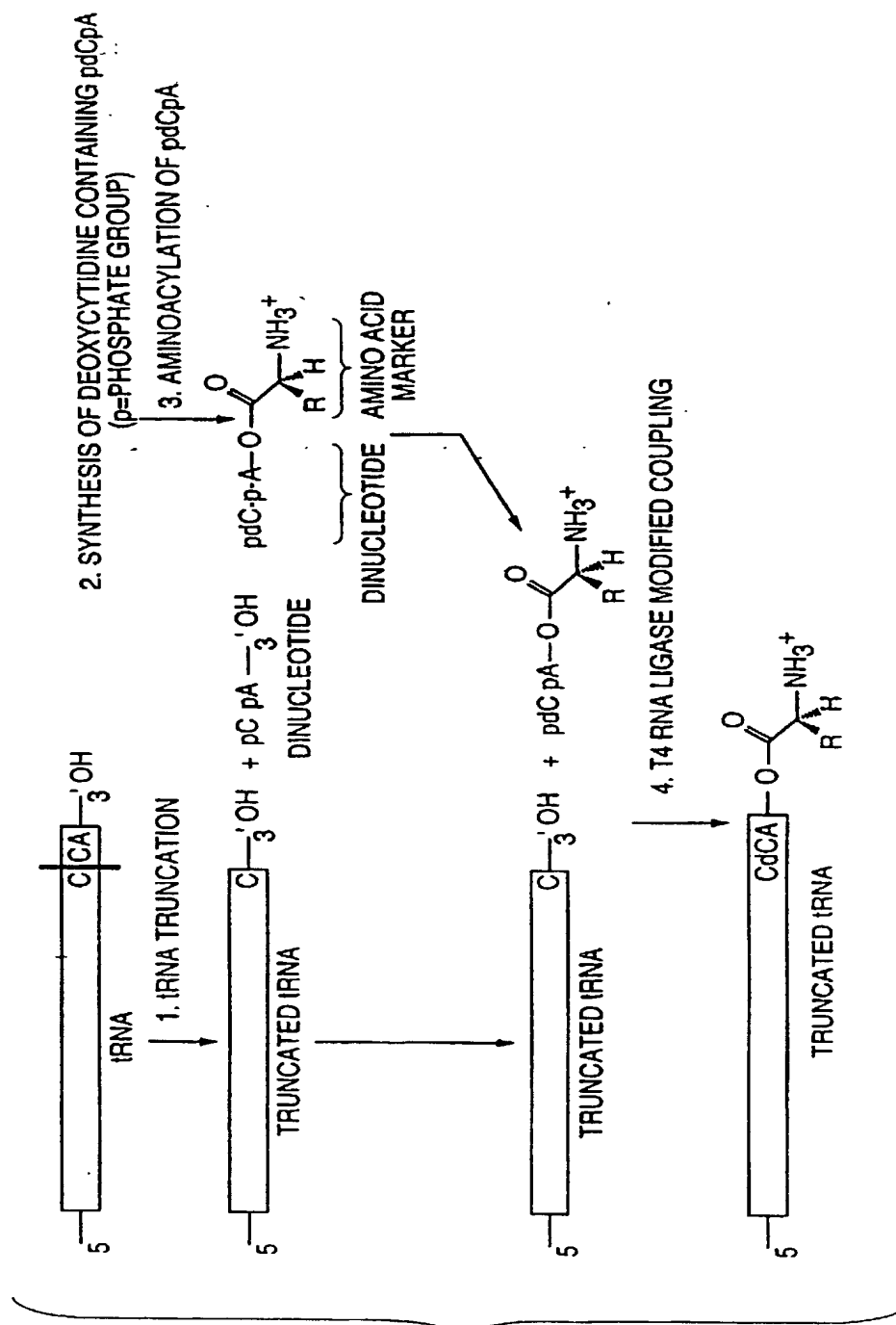


FIG. 16

FIG. 17

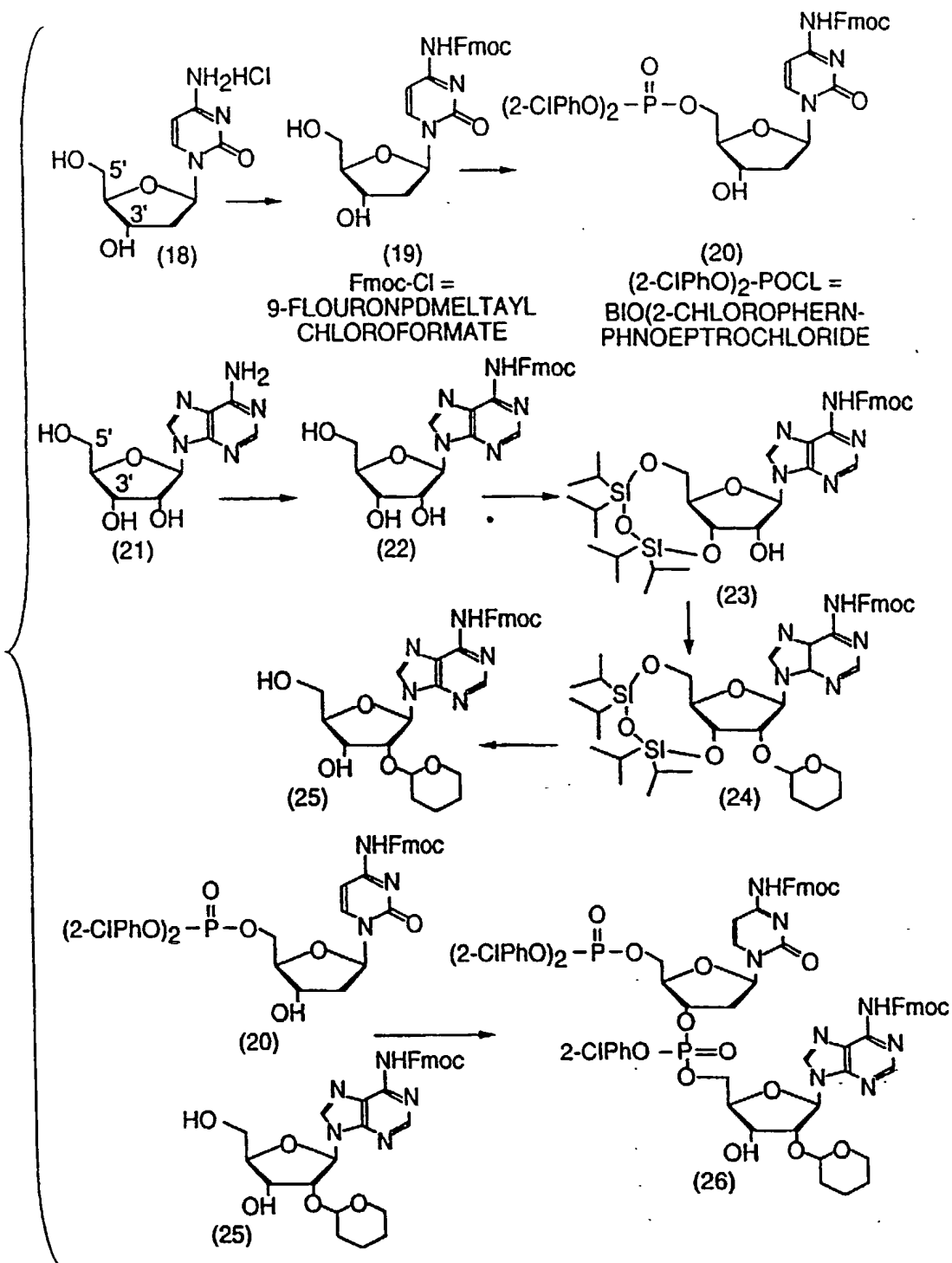
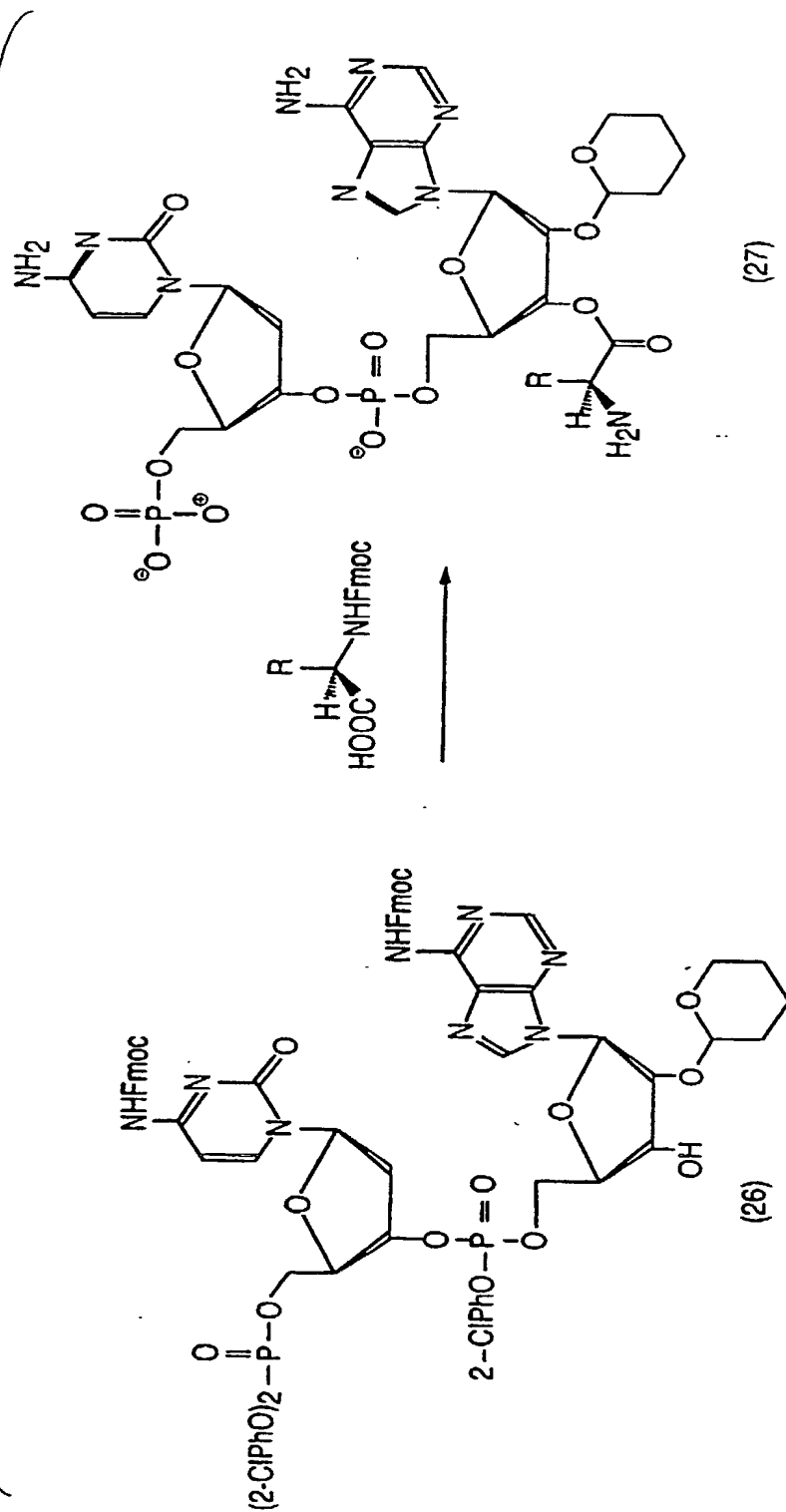


FIG. 18



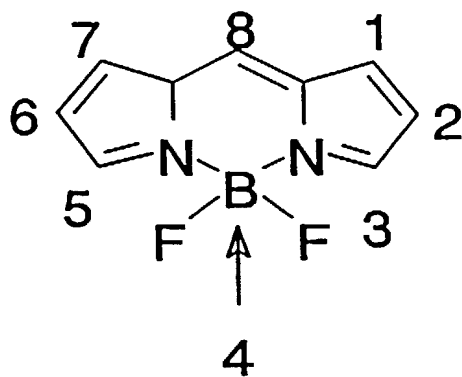
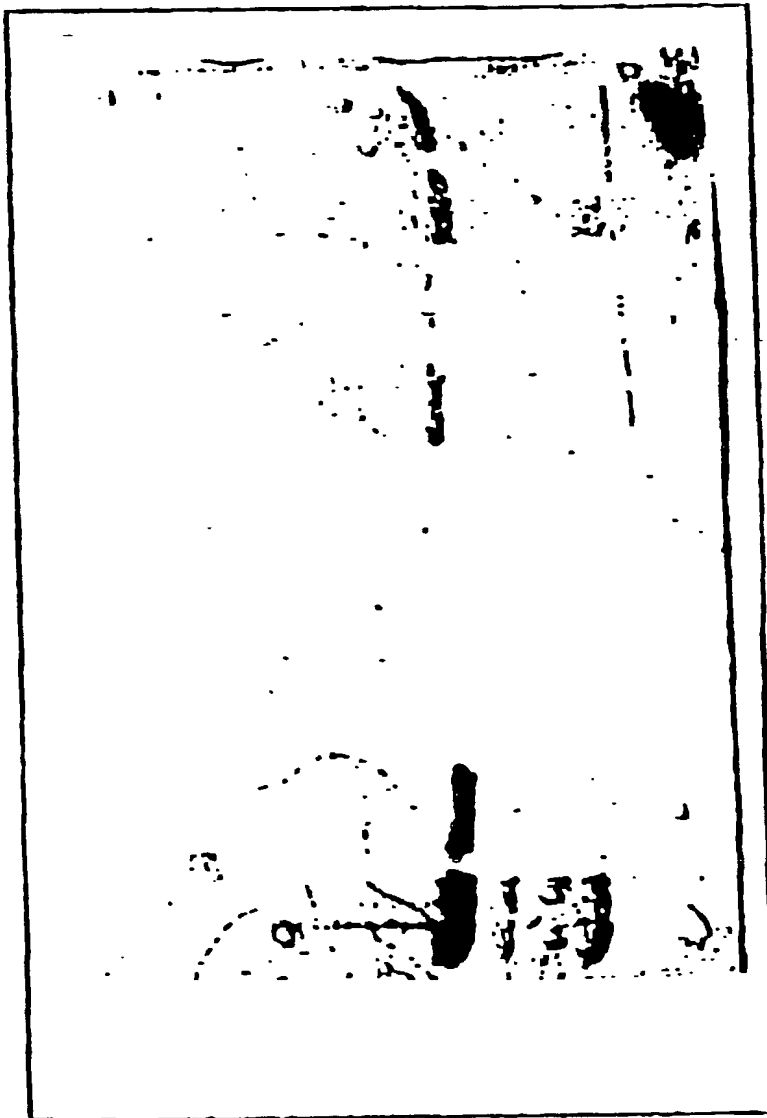


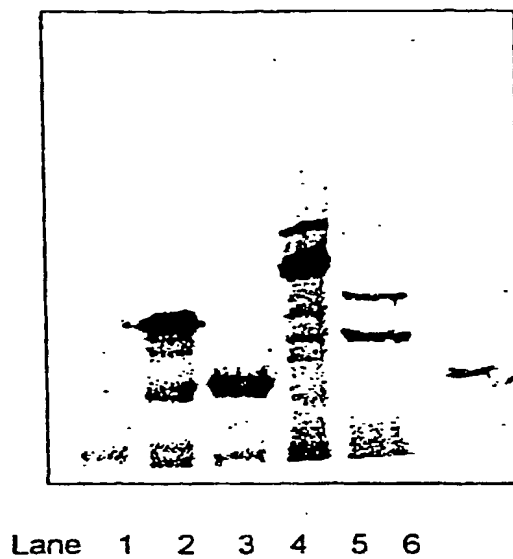
Figure 19



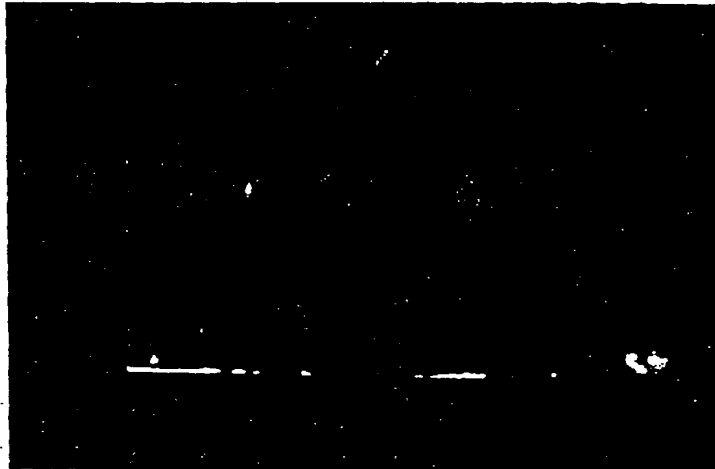


1 2 3 4 5 6 7 8 9 10

**Figure 20**



**Figure 21A**



**Figure 21B**

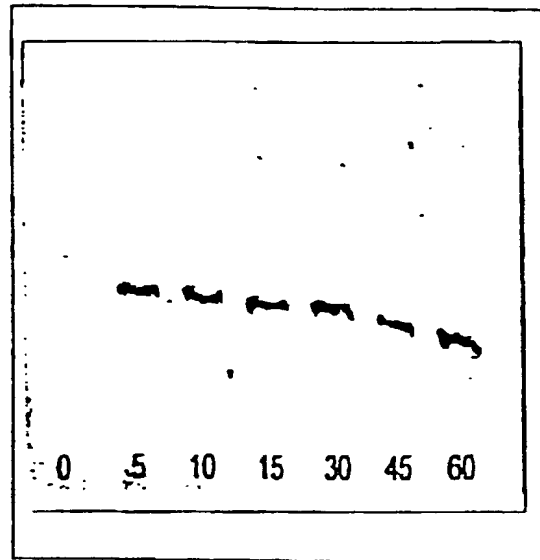
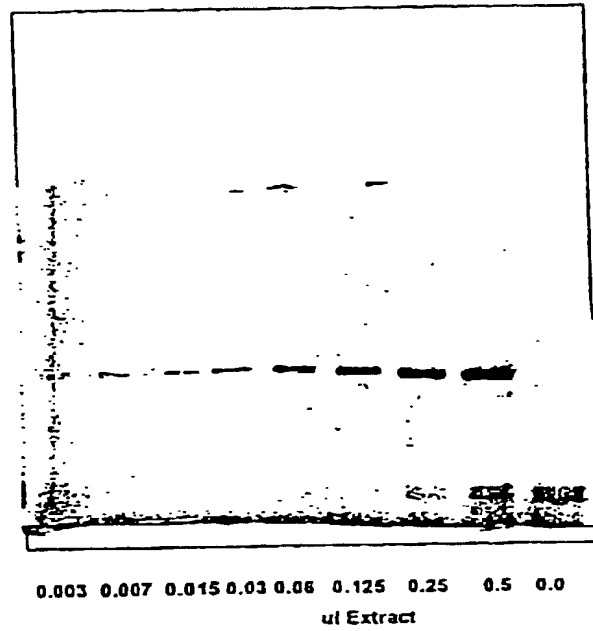
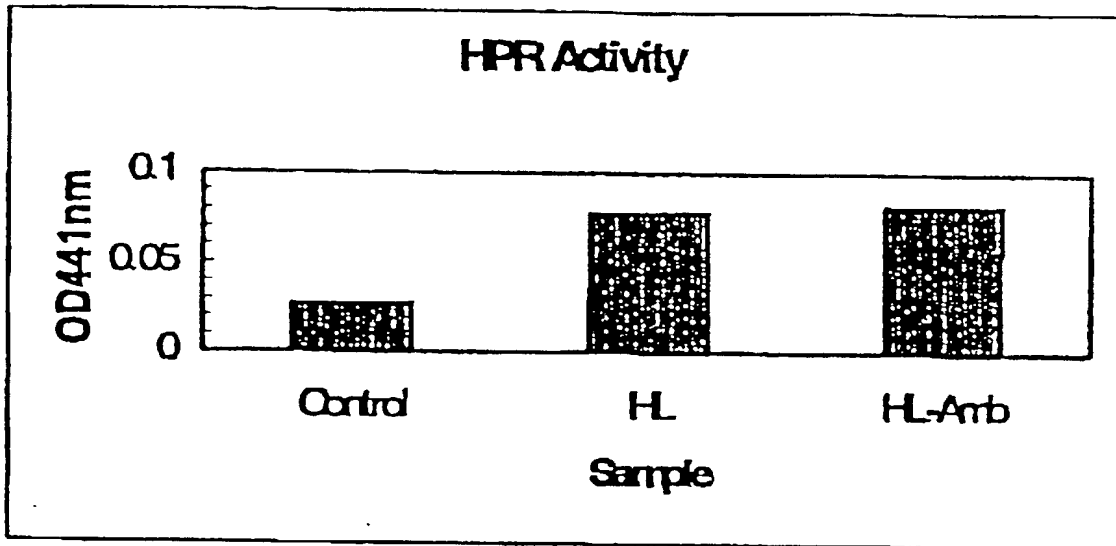


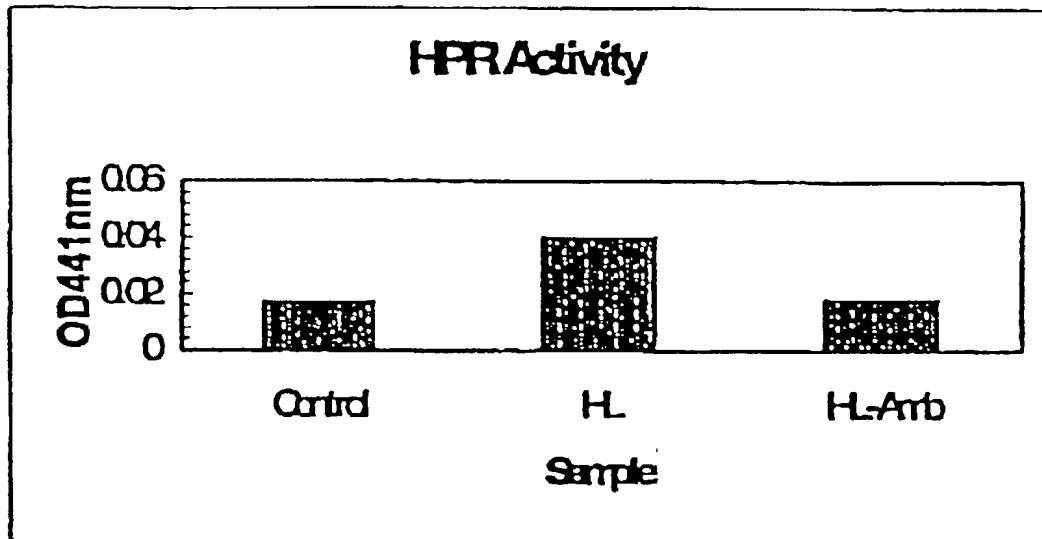
Figure 22A



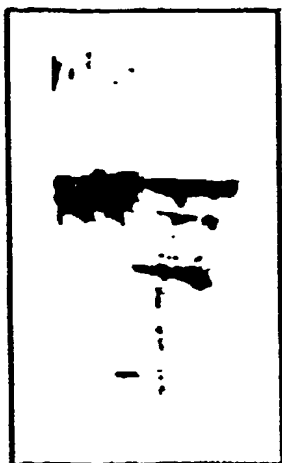
**Figure 22B**



**Figure 23A**



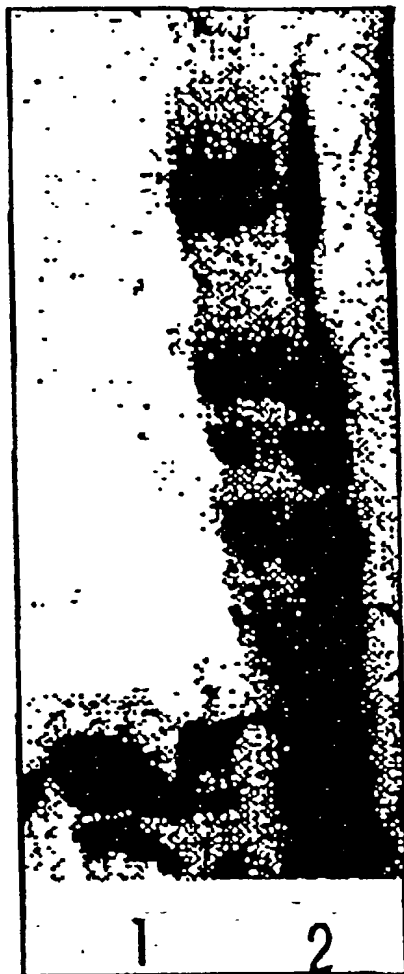
**Figure 23B**



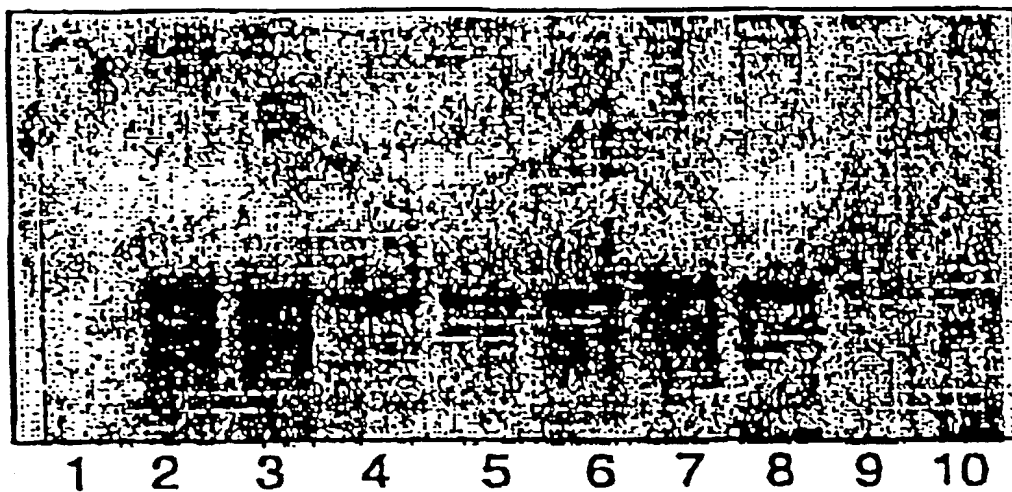
1 2

**Figure 24**

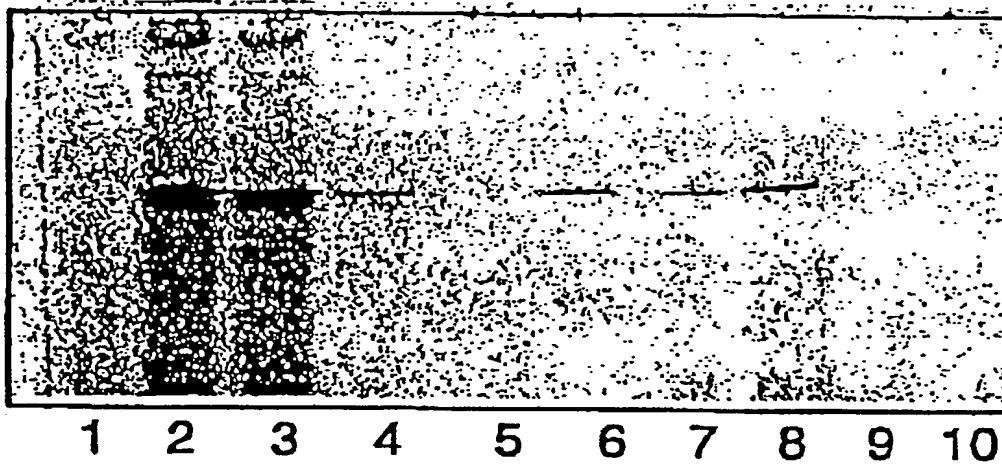




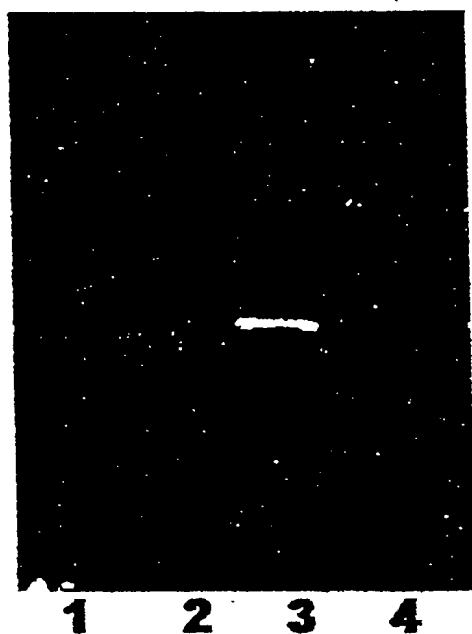
**Figure 25**



**Figure 26A**



**Figure 26B**



**Figure 27**

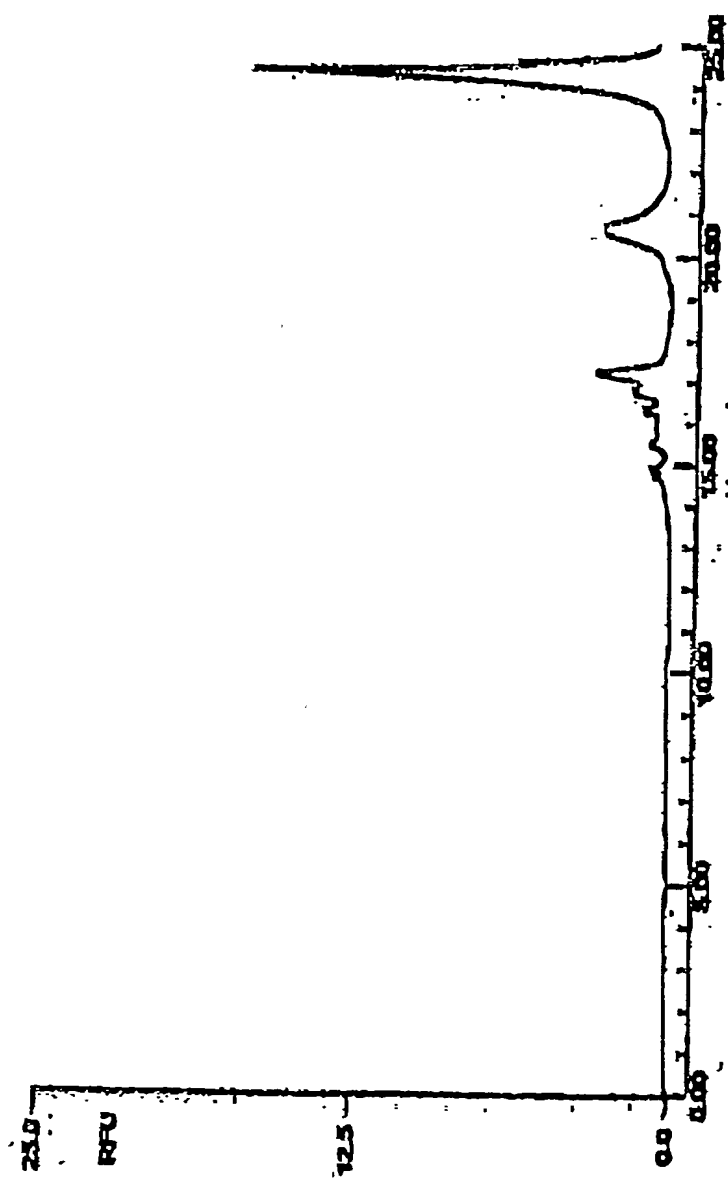


Figure 28A

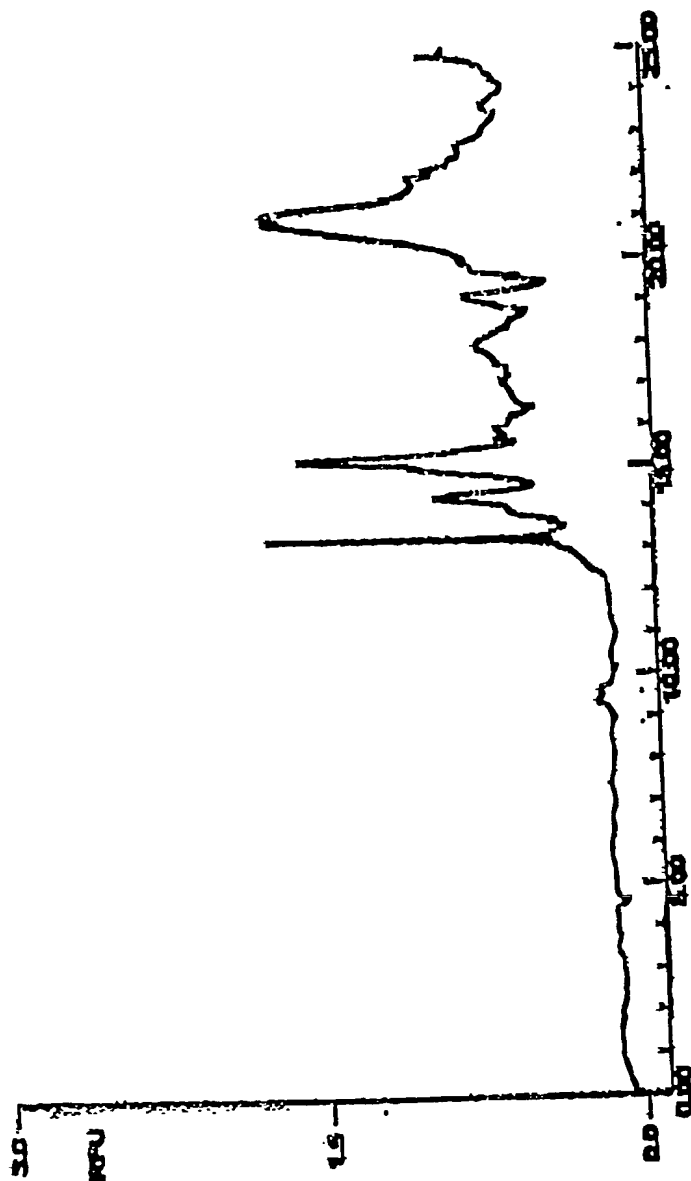


Figure 28B

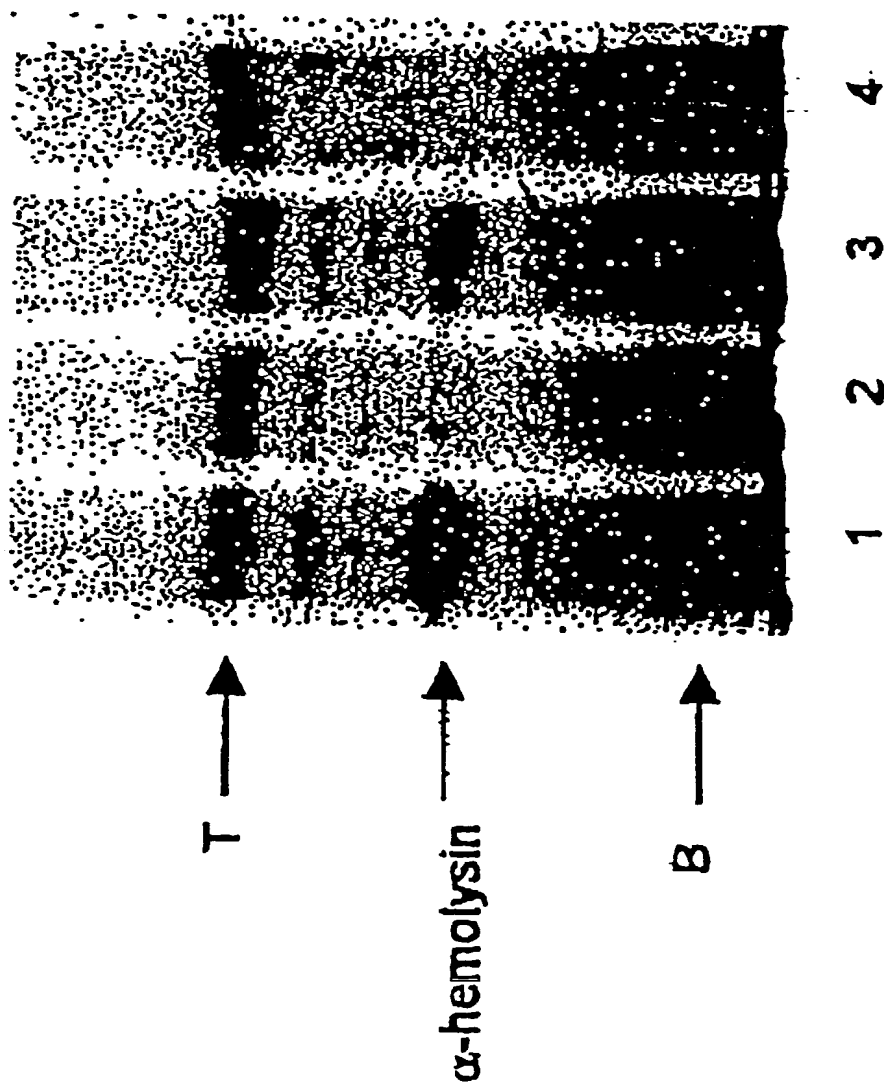
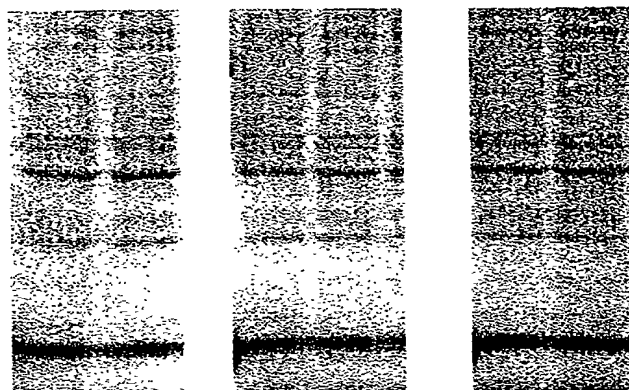


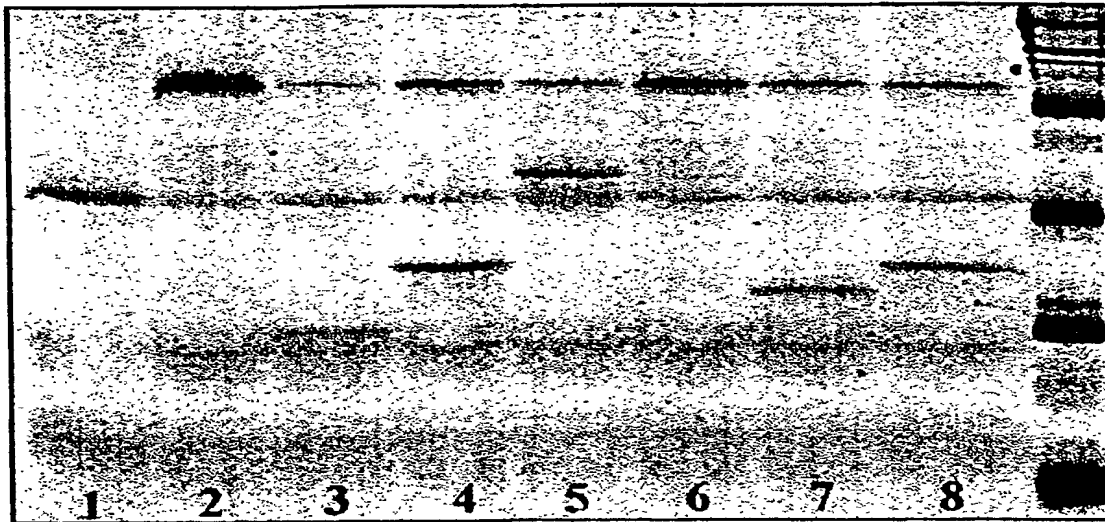
Figure 29



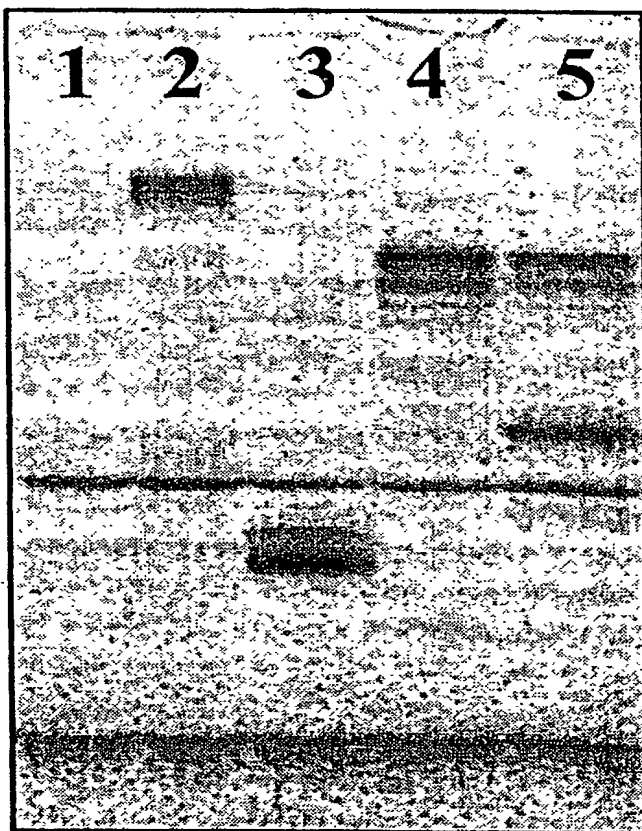
**FIG.30A**



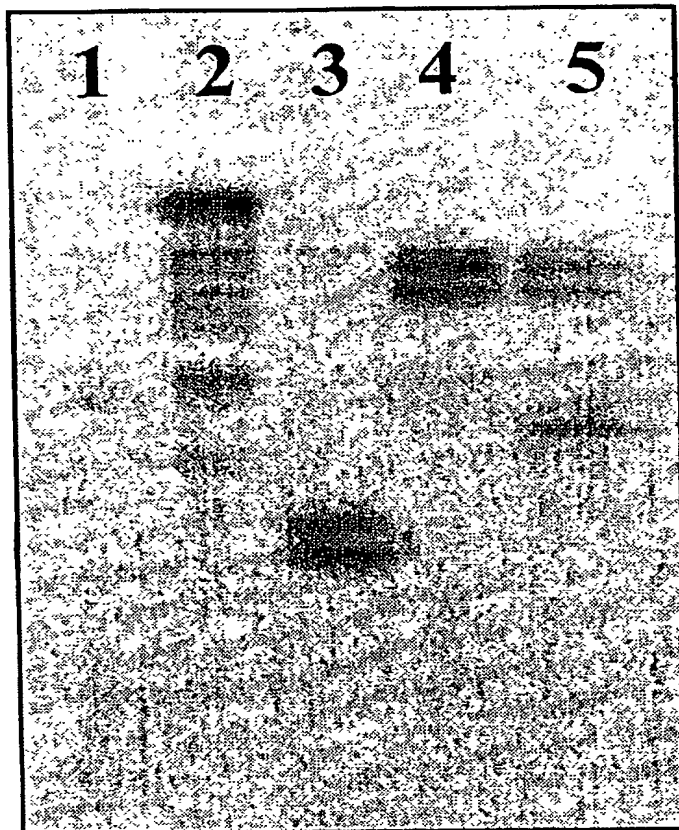
40/53



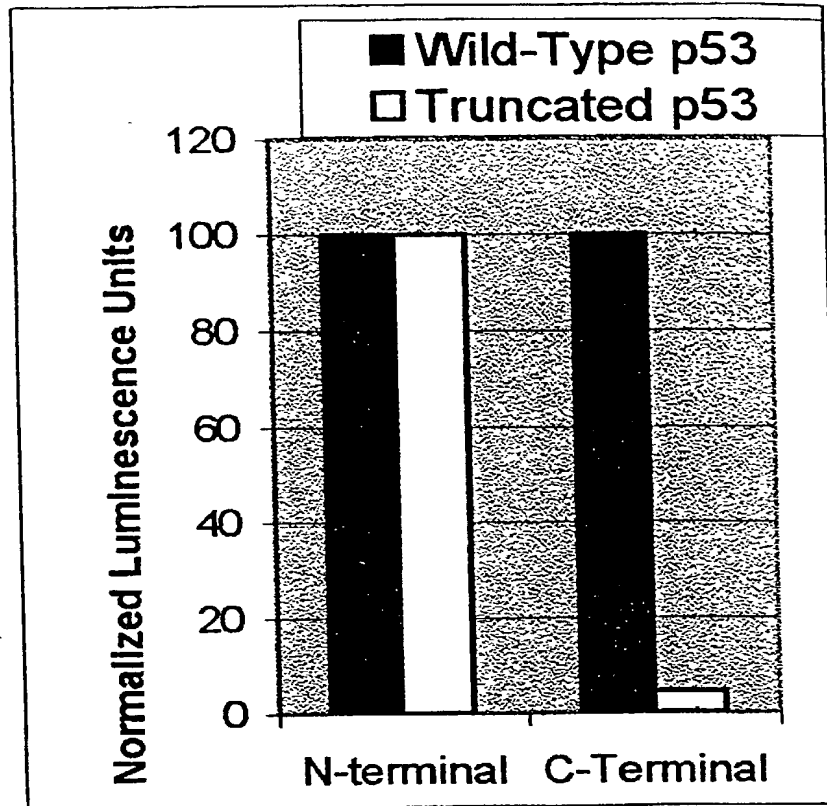
**FIG. 31**



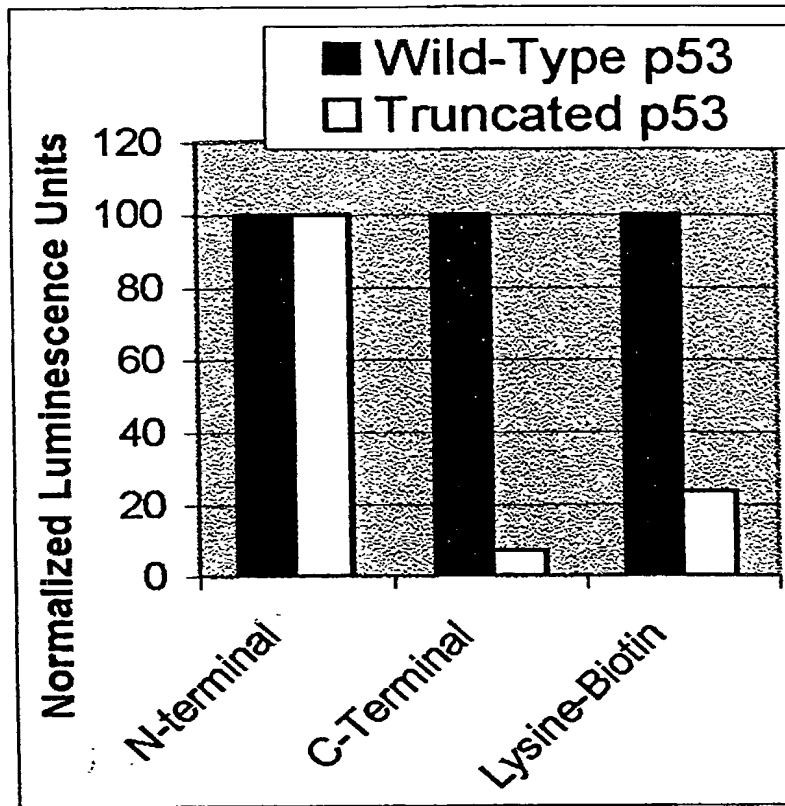
**FIG. 32A**



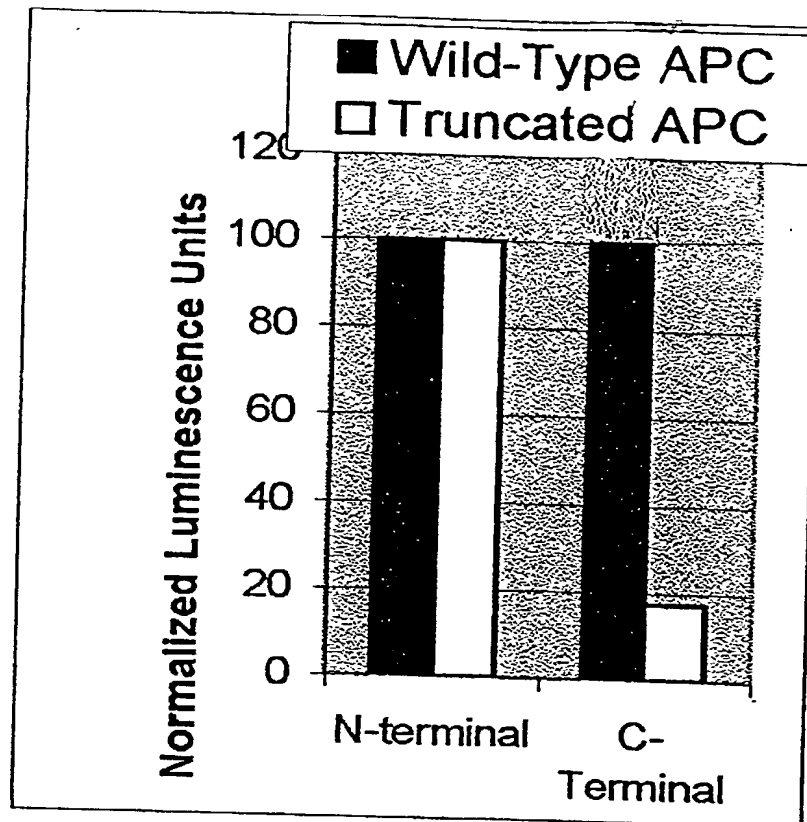
**FIG. 32B**



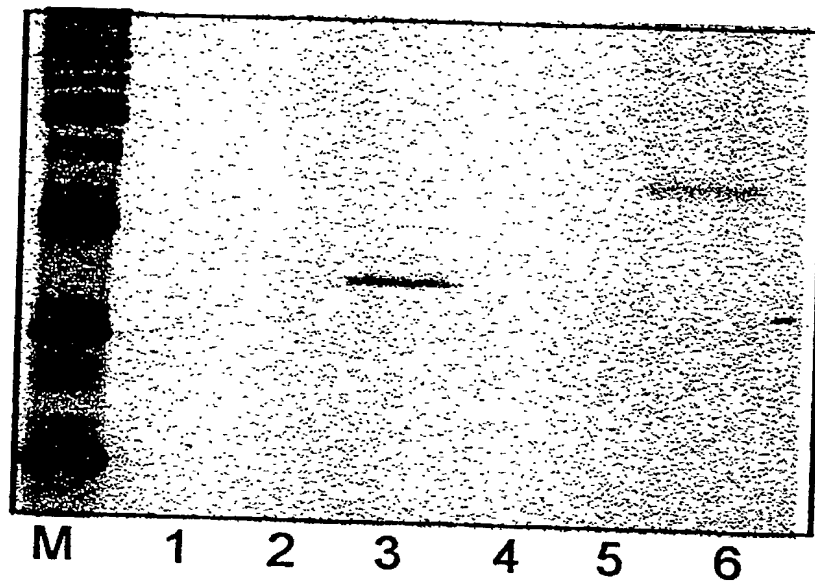
**FIG. 33A**



**FIG. 33B**



**FIG. 33C**



**FIG. 34**



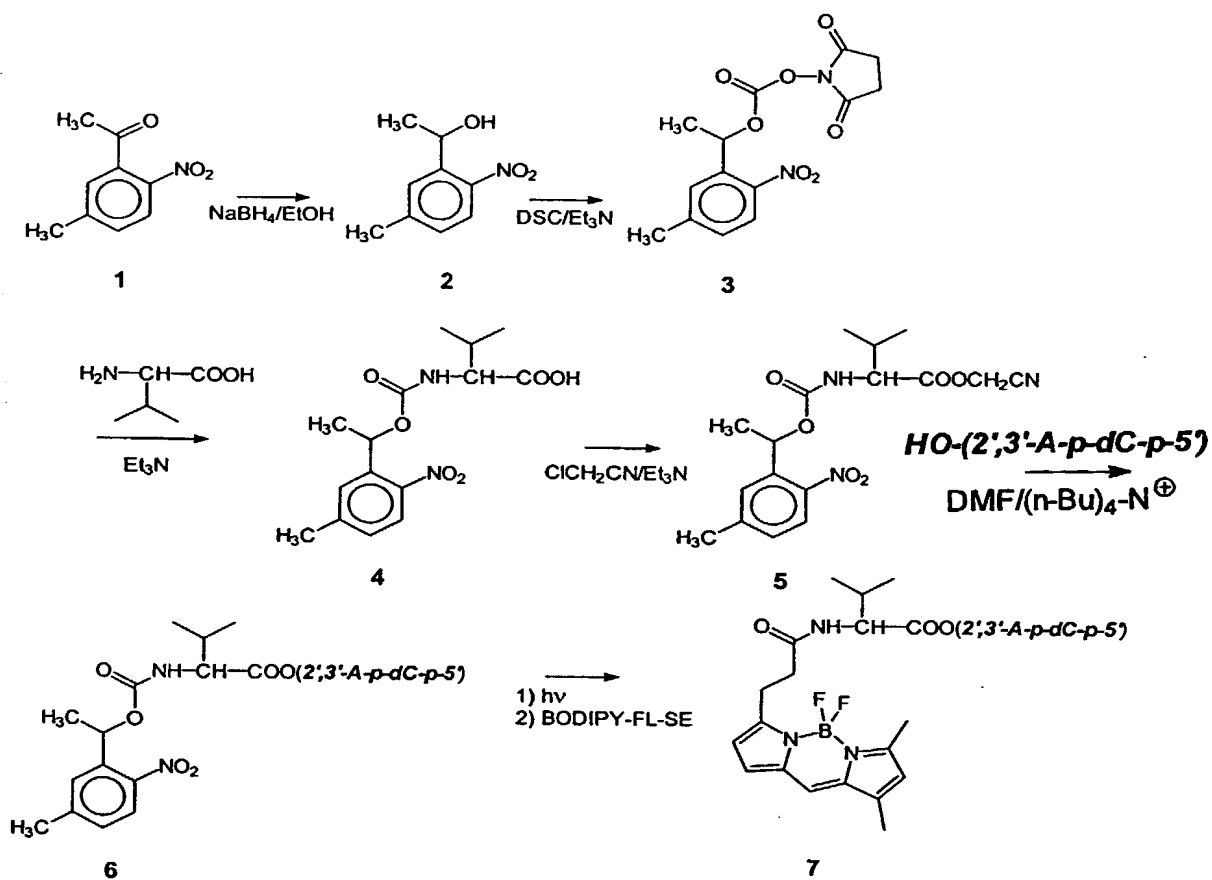
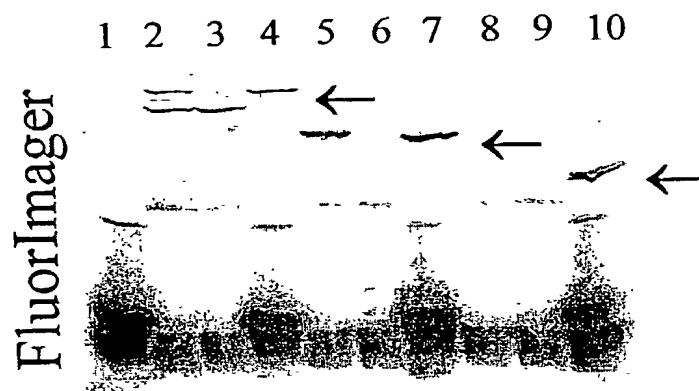
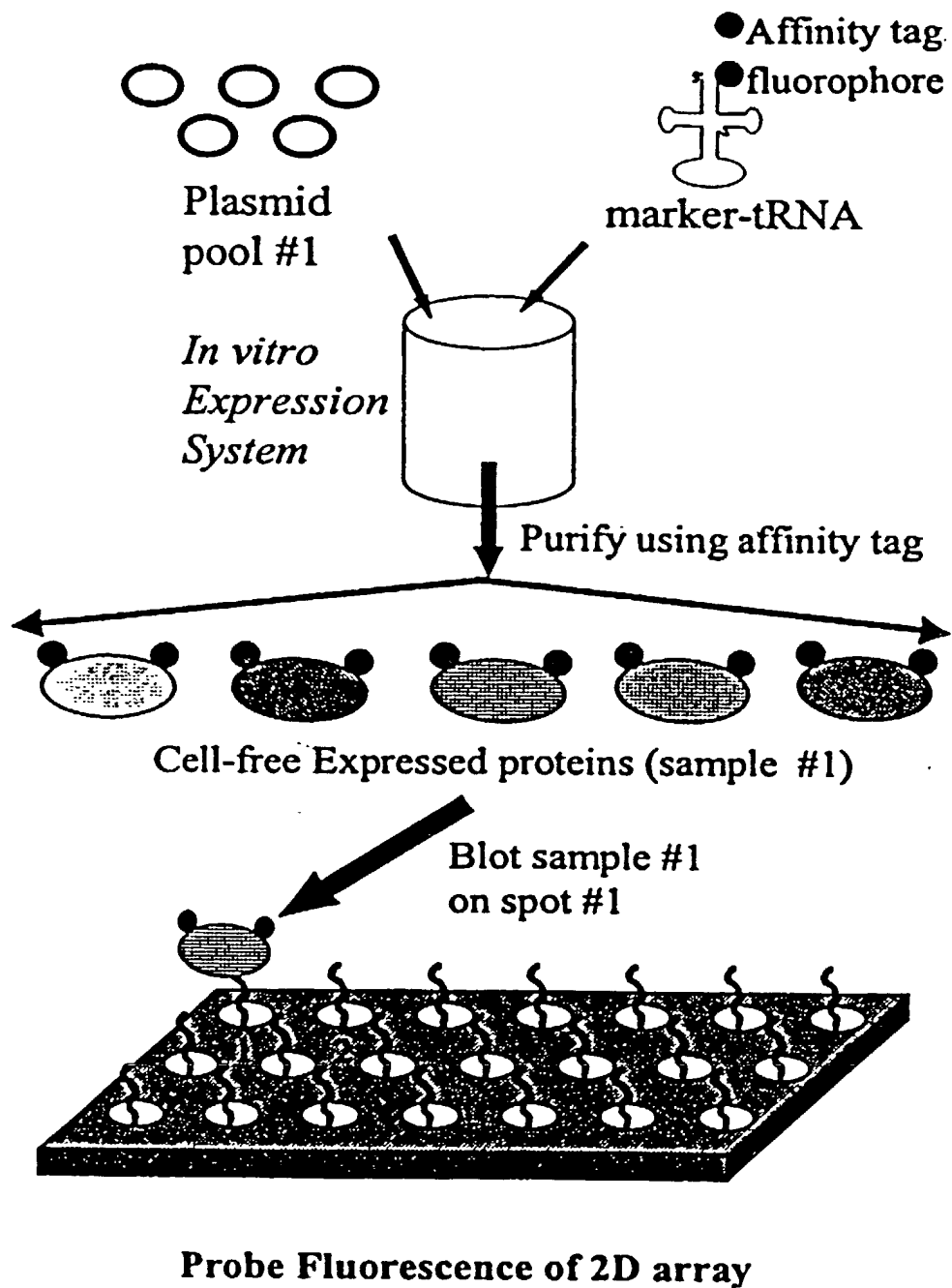


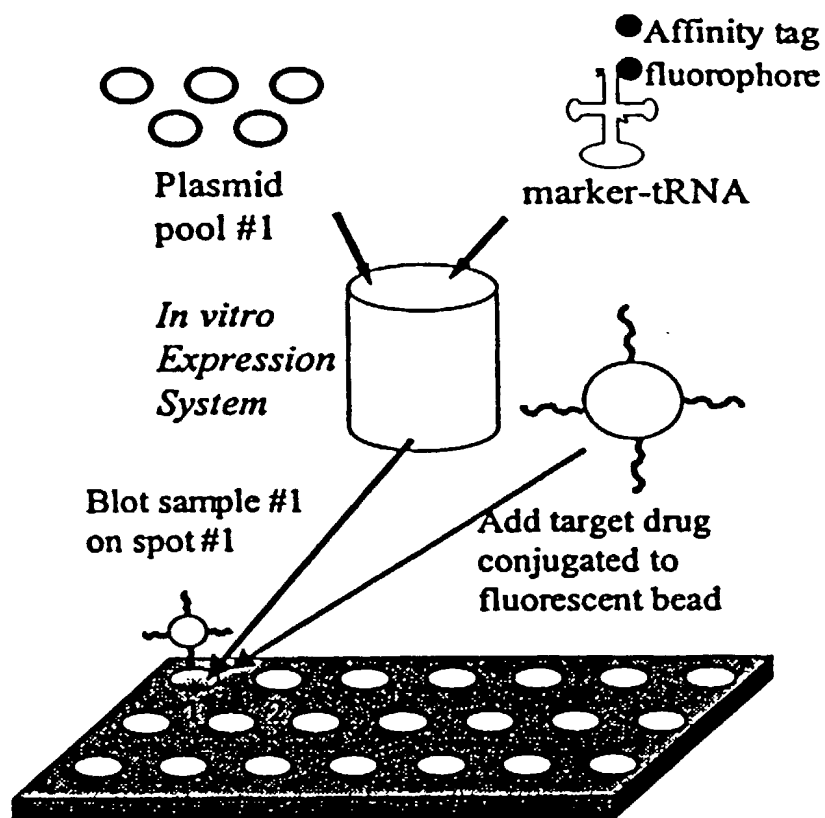
FIG. 35





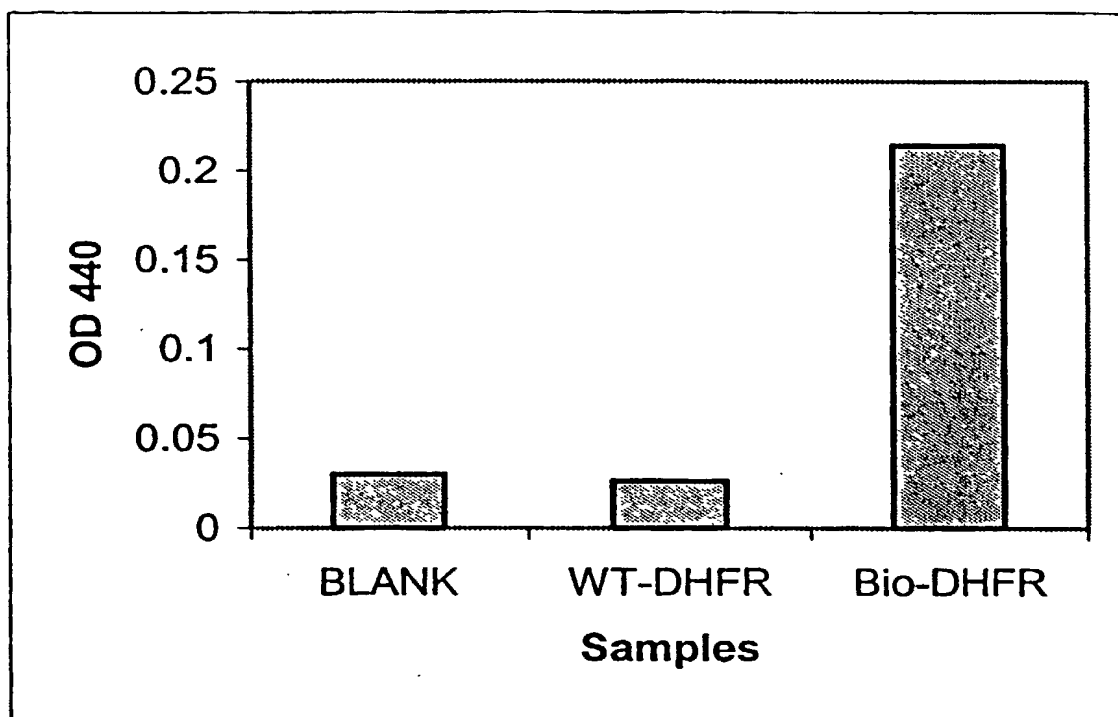
**FIG. 37**

**FIG. 38**



Probe dual Fluorescence of 2D array

**FIG. 39**

**FIG. 40**

Attorney Docket No.: AMBER-06797

TRANSMITTAL LETTER TO THE UNITED STATES  
ELECTED OFFICE (EO/US)  
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II PCT)

1616 Rec'd PCT/PTO

10/049332  
11 FEB 2002

International Appln No.  
PCT/US00/23233

International Filing Date:  
23 August 2000 (23.08.00)

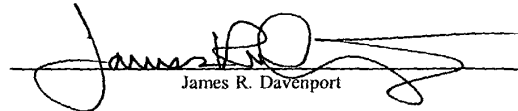
Priority Date Claimed:  
25 August 1999 (25.08.99)

Title of Invention: **Methods for the Detection, Analysis and Isolation of Nascent Proteins**

Applicant: AMBERGEN, INC.

**CERTIFICATION UNDER 37 C.F.R. § 1.10**

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the U.S. Postal Service on this date **February 11, 2002** in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number **EV 008 739 693 US** addressed to: **Box PCT, ATTN: EO/US, Assistant Commissioner For Patents, Washington, D.C. 20231.**

  
James R. Davenport

**BOX PCT**

Assistant Commissioner For Patents  
Washington, D.C. 20231

ATTN: EO/US

1. a. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.  
b.     This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
2. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:
  - a. X This express request to immediately begin national examination procedures (35 U.S.C. 371(f).
  - b. X The U.S. National Fee (35 U.S.C. 371(c)(1) and other fees (37 CFR 1.492) as indicated below:
3. **Fees**  
Regular application

**CLAIMS AS FILED**

Number Filed	Number Extra	Rate	Calculations
Total Claims (37 C.F.R. § 1.16(c))	68 - 20 =	48 × \$18.00 =	\$864.00
Independent Claims (37 C.F.R. § 1.16(b))	7 - 3 =	4 × \$84.00 =	\$336.00
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))	+ \$280.00 =		\$0.00
Filing Fee Calculation			\$1,200.00

X U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY  
Where an international preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO:

X and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) 100.00 100.00  
    and the above requirements are not met (37 CFR 1.492(a)(1)) 690.00

U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY  
 Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO:

—	has been paid (37 CFR 1.492(a)(2))	710.00	_____
—	has not been paid (37CFR 1.492(a)(3)	1000.00	_____
—	where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)	860.00	_____

<b>Total National Fee</b>	<b>1,300.00</b>
<b>Small Entity - Reduction by 1/2, if applicable</b>	<b>650.00</b>
<b>Total Fees Enclosed</b>	<b>650.00</b>

X Check in the amount of \$650.00 to cover the above fees is enclosed.

4. **A copy of the International application as published (35 U.S.C. 371(c)(2)):**

- a. — is transmitted herewith.  
 b. X is not required, as the application was filed with the United States Receiving Office.  
 c. — has been transmitted  
     i. — by the International Bureau.  
        Date of mailing of the application (from form PCT/1B/308): \_\_\_\_  
     ii. — by applicant on (date) \_\_\_\_\_.

5. **A Translation of the International application into the English language (35 U.S.C. 371(c)(2)):**

- a. — is transmitted herewith.  
 b. X is not required as the application was filed in English.  
 c. — was previously transmitted by applicant on (date) \_\_\_\_\_.  
 d. — will follow.

6. **Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):**

- a. — are transmitted herewith (as a part of the International Examination Report)  
 b. — have been transmitted  
     i. — by the International Bureau.  
        Date of mailing of the amendment (from form PCT/1B/308): \_\_\_\_\_.  
     ii. — by applicant on (date) \_\_\_\_\_.  
 c. X have not been transmitted as  
     i. X applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210.): 14 November 2000 (14.11.00).  
     ii. — the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

7. **A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):**

- a. — is transmitted herewith.  
 b. — is not required as the amendments were made in the English language.  
 c. X has not been transmitted for reasons indicated at point 5c above.

8. **A copy of the international examination report (PCT/IPEA/409)**

- is transmitted herewith.  
X is not required as the application was filed with the United States Receiving Office.

9. **Annex(es) to the international preliminary examination report**

- a. — is/are transmitted herewith.  
 b. X is/are not required as the application was filed with the United States Receiving Office.



10. A translation of the annexes to the international preliminary examination report **JC13 Rec'd PCT/PTO 11 FEB 2002**

- a. ☐ is transmitted herewith.  
b. ☒ is not required as the annexes are in the English language.

11. An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115

- a. ☐ was previously submitted by applicant on (date) \_\_\_\_\_.  
b. ☐ is submitted herewith, and such oath or declaration  
i. ☐ is attached to the application.  
ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3b or 3c and 5b; and states that they were reviewed by the inventor as required by 37 CFR 1.70.  
iii. ☒ will follow (unexecuted declaration is enclosed).

II. Other document(s) or information included:

12. An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

- a. ☐ is transmitted herewith.  
b. ☐ has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): \_\_\_\_\_  
c. ☒ is not required, as the application was searched by the United States International Searching Authority.  
d. ☐ will be transmitted promptly upon request.  
e. ☐ has been submitted by applicant on (date) \_\_\_\_\_

13. An Information Disclosure Statement under 37 CFR 1.97 and 1.98:

- a. ☐ is transmitted herewith.  
Also transmitted herewith is/are:  
☐ Form PTO-1449.  
☐ Copies of citations listed.  
b. ☒ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).  
c. ☐ was previously submitted by applicant on (date) \_\_\_\_\_

14. An Assignment document:

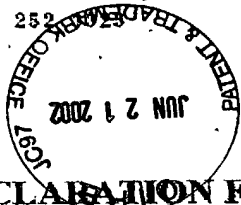
- a. ☒ will follow (an unexecuted assignment is enclosed).  
b. ☐ is transmitted herewith for recording.  
A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

15. Additional Documents

- a. ☐ Copy of Request (PCT/RO/101)  
b. ☒ International Publication No. WO/01/14578  
i. ☐ Specification, claims and drawing  
ii. ☒ Front page only.  
c. ☐ Preliminary amendment (37 CFR § 1.121)  
d. ☒ Other  
Small Entity Declaration (unexecuted)  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

16. The above checked items are being transmitted

- a. ☒ before 30 months from any claimed priority date.  
b. ☐ after 30 months.



PATENT

Attorney Docket No.: AMBER-06797

## DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **Methods For The Detection, Analysis And Isolation Of Nascent Proteins**, the specification of which is attached hereto. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

<u>PCT/US00/23233</u>	<u>08/23/00</u>	<u>Yes</u>
<i>Number</i>	<i>Day/Month/Year Filed</i>	<i>Priority Claimed</i>
I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:		
<u>09/382,950</u>	<u>08/25/99</u>	<u>Patented</u>
<u>09/382,736</u>	<u>08/25/99</u>	<u>Patented</u>
<i>Application Serial No.</i>	<i>Filing Date</i>	<i>Patented, Pending or Abandoned</i>

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

100 Full Name of Sole or First Inventor: Kenneth J. Rothschild

Inventor's Signature: [Signature]

Residence: 97 Dorcar Road, Newton, Massachusetts 02139

Post Office Address: 97 Dorcar Road, Newton, Massachusetts 02139

Date: 6/04/2002

Citizenship: United States of America

200 Full Name of Second Joint Inventor: Sadanand Gite

Inventor's Signature: [Signature]

Residence: 808 Memorial Drive, Cambridge, Massachusetts 02139

Post Office Address: 808 Memorial Drive, Cambridge, Massachusetts 02139

Date: 5/25/02

Citizenship: India

300 Full Name of Third Joint Inventor: Jerzy Olejnik

Inventor's Signature: [Signature]

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Date: 5/23/02

Citizenship: Poland

## PATENT

Attorney Docket No.: AMBER-06797

400 Full Name of Fourth Joint Inventor: Sergey MarnayevInventor's Signature: *Sergey Marnayev*Date: 05.23.02Residence: 2 Franclaire Drive, West Roxbury, Massachusetts 02132Citizenship: RussiaPost Office Address: 2 Franclaire Drive, West Roxbury, Massachusetts 02132

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500 Full Name of Fifth Joint Inventor: Mark LinnInventor's Signature: *Mark Linn*Date: 5/23/02Residence: 85 Sutherland Road, Brookline, Massachusetts 02446Citizenship: United States of AmericaPost Office Address: 85 Sutherland Road, Brookline, Massachusetts 02446

MA

10/049332

17. Certain Requirements under 35 U.S.C. 371 were previously submitted by the applicant  
on \_\_\_\_\_, namely:

JC13 Rec'd PCT/PTO 11 FEB 2002

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\_\_\_\_\_


18. Authorization To Charge Additional Fees and Credit Overpayment

X The Commissioner is hereby authorized to charge payment of any additional fees that may be required by this paper including, without limitation, filing fees, fees for presentation of extra claims, and surcharge for filing late declarations to Deposit Account No.: 08-1290.  
An originally executed duplicate of this transmittal is enclosed for this purpose.

19. Return Receipt Postcard

X Enclosed.

Dated: February 11, 2002

  
MACKNIGHT, Kamrin T.  
Registration No.: 38,230

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